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JACQUES LOEB W. J. V. OSTERHOUT

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CORRECTION.

In addition to the Correction in Vol. iii, No. 3, January 20, 1921, on page 149, Vol. iii, No. 2, November 20, 1920, line 13, for *92.57* read *89.10*; page 154, lines 15, 28, and 32, for *O* read *O+10*; page 155, line 3 of the figure legend, for *O* read *O+10*.

A THEORY OF INJURY AND RECOVERY.

II. Experiments with Mixtures.

By W. J. V. OSTERHOUT.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

(Received for publication, January 15, 1921.)

The electrical conductivity of *Laminaria* changes when the plant is transferred from sea water to 0.52 M NaCl, or to 0.278 M $CaCl_2$, and is subsequently replaced in sea water. It has been found that these changes can be predicted with considerable accuracy by means of certain equations. According to the theory of the writer,[1] the same equations should enable us to predict the changes produced by transferring tissue from sea water to mixtures of NaCl and $CaCl_2$ and then replacing the plant in sea water.

In order to test this theory experiments were made with a variety of mixtures. The solutions employed are given in Table I. The electrical resistance of the tissue in these solutions is shown in Fig. 1.

The experimental data are in good agreement with the values calculated by means of the formula:

$$\text{Resistance} = 2{,}700\left(\frac{K_A}{K_M - K_A}\right)\left(e^{-K_A T} - e^{-K_M T}\right) + 90\, e^{-K_M T} + 10$$

This is evident from Fig. 1, which shows the curves calculated by means of this formula and also the observed values.[2]

[1] Osterhout, W. J. V. *J. Gen. Physiol.*, 1920–21, iii, 145. For corrections see the slips inserted in this and in the preceding number of the *Journal*.

[2] The points shown in Fig. 1 (except those for the solutions containing 1.41 and 2.44 per cent $CaCl_2$) are taken from Fig. 1 of a previous paper (cited below), but the curves here given are not identical with those shown in that figure since the curves of the present paper represent the calculated values (using the velocity constants given in Table II), while those in Fig. 1 of the former paper are merely smoothed curves drawn through the observed points.

The results described in the former paper as obtained with solutions containing

TABLE I.

Composition of Mixtures.

0.52 M NaCl	0.278 M $CaCl_2$	Molecular proportions in the mixture.	
		NaCl	$CaCl_2$
cc.	*cc.*	*per cent*	*per cent*
973	27	98.59	1.41
955	45	97.56	2.44
914	86	95.24	4.76
751	249	85.00	15.00
496	504	65.00	35.00
247	753	38.00	62.00

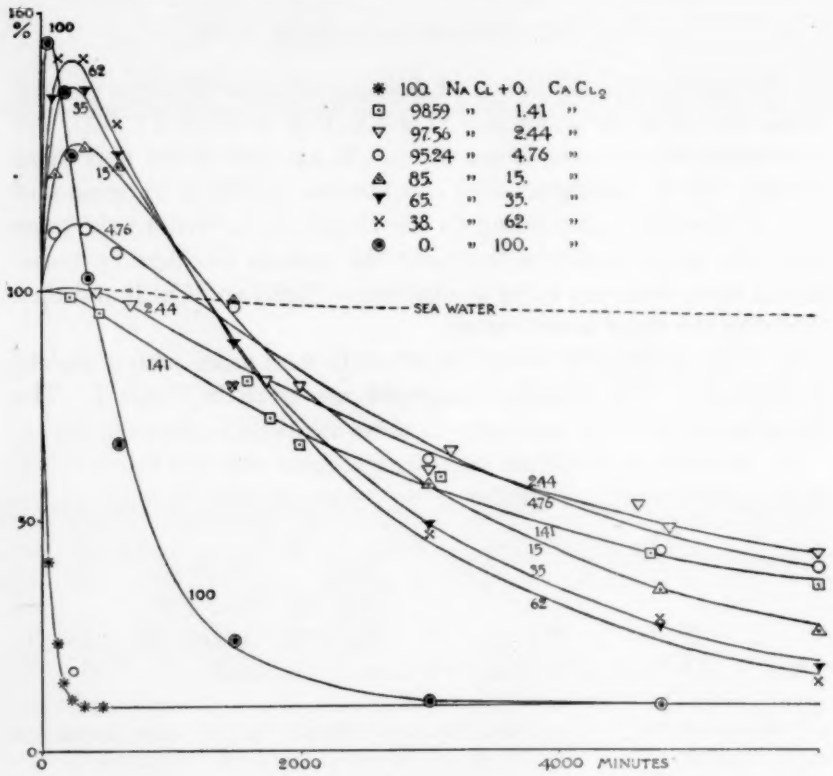

FIG. 1. Curves showing the electrical resistance of *Laminaria agardhii* in 0.52 M NaCl, in 0.278 M $CaCl_2$, and in mixtures of these (the figures attached to the curves show the molecular per cent of $CaCl_2$ in the solution). The curves show the calculated values (from constants obtained by trial, which are given in Table II); the points show the observed values (some are omitted in order to avoid undue crowding); each represents the average of six or more experiments. Probable error of the mean less than 10 per cent of the mean.

This formula is based upon the assumption that the electrical resistance is proportional to a substance, M, which is formed and decomposed by the reactions[3]

$$O \rightarrow S \rightarrow A \rightarrow M \rightarrow B$$

We assume that when the tissue is transferred from sea water to NaCl, or to $CaCl_2$, or to a mixture of these two solutions, the reactions $O \rightarrow S \rightarrow A$ cease, while the reactions $A \rightarrow M \rightarrow B$ continue. By assuming various values of K_A (the velocity constant of the reaction $A \rightarrow M$) and of K_M (the velocity constant of the reaction $M \rightarrow B$), and employing these in the formula, we obtain curves which closely approximate those which we find by experiment. The values of the velocity constants which are thus obtained are given in Table II.

It is evident from Table II that as the per cent of $CaCl_2$ in the mixtures increases (beginning at 1.41 per cent $CaCl_2$) the value of K_M first falls and then rises, its minimum value occurring in 97.56 NaCl + 2.44 $CaCl_2$ (which is the mixture in which the tissue lives the longest). It seems reasonable to assume that in each mixture a substance is formed which reduces the value of K_M. We may assume that the decrease of K_M is directly proportional to the amount of this substance, which may be assumed to occur in maximum amount in 97.56 NaCl + 2.44 $CaCl_2$.

The simplest assumption which we can make is that NaCl and $CaCl_2$ combine with some constituent of the protoplasm, as XZ_2, to form a compound.[4] If we suppose that the compound is Na_4XCa, formed by the reversible reaction

$$4\,NaCl + XZ_2 + CaCl_2 \rightleftharpoons Na_4XCa + 2\,ZCl_2$$

we can calculate the amount of Na_4XCa which is formed in each mixture of NaCl and $CaCl_2$.

2 per cent $CaCl_2$ are omitted, as it was subsequently found that this solution contained more than 2 per cent $CaCl_2$.

For the data concerned and for the derivation of the formula see Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533. In the present paper the formula previously used is multiplied by 305 and 10 is added.

[3] These are regarded as monomolecular and irreversible or practically so.

[4] It is assumed that XZ_2, Na_4XCa, and ZCl_2 are in solution. Since the per cent of XZ_2 which is transformed to Na_4XCa is negligible, the concentration of XZ_2 may be regarded as constant.

TABLE II.

Velocity Constants at 15°C.

Reaction.	Velocity constant.	Value of the velocity constant in*							
		NaCl	NaCl 98.59 $CaCl_2$ 1.41	NaCl 97.56 $CaCl_2$ 2.44	NaCl 95.24 $CaCl_2$ 4.76	NaCl 85.0 $CaCl_2$ 15.0	NaCl 65.0 $CaCl_2$ 35.0	NaCl 38.0 $CaCl_2$ 62.0	$CaCl_2$
$N \rightarrow O$	K_N	0.03	0.00046	0.0004	0.00061	0.00091	0.0013	0.00134	0.005033
$O \rightarrow P$	K_O	0.0297	0.0004554	0.000396	0.0006039	0.0009009	0.001287	0.0013266	0.0049827
$R \rightarrow S$	K_R	0.04998	0.0005623	0.00056	0.001055	0.001606	0.0026	0.003509	0.012532
$S \rightarrow T$	K_S	0.02856	0.0008048	0.000874	0.00151	0.0017446	0.00254	0.00319	0.010848
$A \rightarrow M$	K_A	0.018	0.000222	0.000187	0.000245	0.000364	0.000481	0.00053	0.0018
$M \rightarrow B$	K_M	0.540	0.00666	0.00546	0.00590	0.00730	0.00859	0.009	0.0295

* The figures at the heads of columns refer to molecular proportions in the mixture.

For reasons given in a former paper,[5] we assume that this reaction occurs at the surface of the cell and that $CaCl_2$ accumulates in the surface to a greater degree than NaCl. The increase in concentration of $CaCl_2$ in the surface is supposed to be ten times as great as the corresponding increase of NaCl, so that the proportions in the surface are those given in Table III. For example, when the proportions in the solution are 97.56 NaCl + 2.44 $CaCl_2$, the proportion of NaCl to $CaCl_2$ in the surface is as 97.56 to 24.40, which is equivalent[6] to 80 NaCl + 20 $CaCl_2$.

TABLE III.

Amount of Na_4XCa.

Molecular proportions.				Amount of Na_4XCa	Increase in Na_4XCa
In the solution.		In the surface.			
NaCl	$CaCl_2$	NaCl	$CaCl_2$		
per cent	*per cent*	*per cent*	*per cent*		
100.0	0	100.0	0	0	0
98.59	1.41	87.5	12.5	0.000902	0.000855
97.56	2.44	80.0	20.0	0.000936	0.000889
95.24	4.76	66.67	33.33	0.000870	0.000823
85.0	15.0	36.27	63.73	0.000488	0.000441
65.0	35.0	15.66	84.34	0.000177	0.000125
38.0	62.0	5.78	94.22	0.000047	0
0	100.0	0	100.0	0	0

We calculate the amount of Na_4XCa by the usual formula:

$$K = \frac{(C_{Na_4XCa})\,(C_{ZCl_2})^2}{(C_{NaCl})^4\,(C_{CaCl_2})\,(C_{XZ_4})}$$

but since $2C_{Na_4XCa} = C_{ZCl_2}$ we may write

$$K = \frac{(C_{Na_4XCa})\,(2\,C_{Na_4XCa})^2}{(C_{NaCl})^4\,(C_{CaCl_2})\,(C_{XZ_4})}$$

[5] Osterhout, W. J., V., *Proc. Am. Phil. Soc.*, 1916, lv, 533.

[6] As explained in a former paper,[5] it is assumed that the reaction takes place in a surface which is saturated with respect to NaCl and $CaCl_2$, so that while one of these may be displaced by the other (in case their relative proportions in the solution are altered) the total concentration does not change; for convenience this concentration is taken as 100 and the sum of NaCl + $CaCl_2$ is therefore always equal to 100.

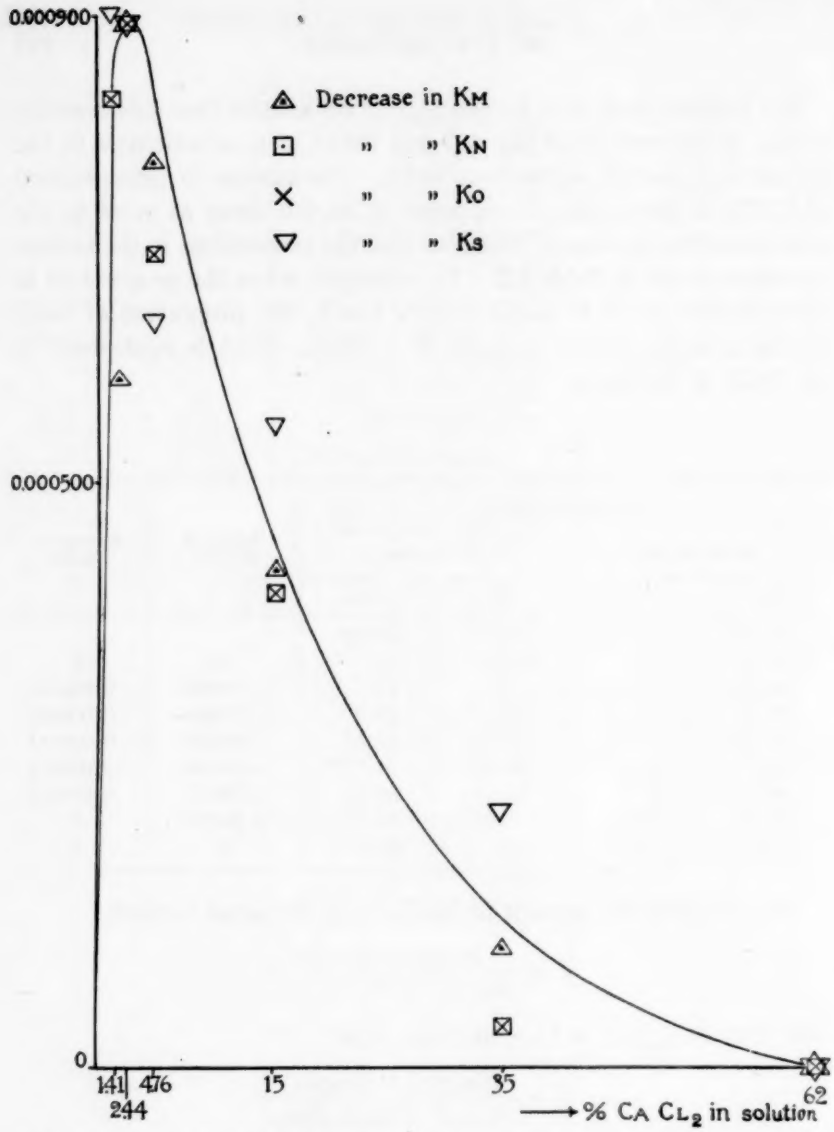

FIG. 2. Curve showing the increase of a hypothetical salt compound Na_4XCa (see Table III); and the corresponding decrease of the velocity constants K_N, K_O, K_S, and K_M (these constants are given in Table II). The figures on the abscissa give the molecular per cent of $CaCl_2$ in the mixture. The mixture containing 62.0 per cent $CaCl_2$ is taken as the standard of comparison: proceeding from this to the mixtures containing less $CaCl_2$ we find that Na_4XCa increases and the velocity constants decrease as shown by the ordinates. In order to facilitate comparison the values of K_N have been multiplied by 0.989; of K_O by 0.991; of K_S by 0.384; and of K_M by 0.251.

Putting $K = 4(10^{-17})$ and $C_{XZ_2} = 0.1$ we get[4]

$$10^{-17} = \frac{(C_{Na_4XCa})^3}{(80)^4\ (20)\ (0.1)}$$

whence $C_{Na_4XCa} = 0.000936$.

Proceeding in the same manner with the other mixtures we get the values given in Table III. Starting with the lowest value (that in 62.0 per cent $CaCl_2$) we observe that there is an increase as the per cent of $CaCl_2$ decreases until 2.44 per cent is reached (the amount of this increase is shown in Column 6 of the table). Conversely we find (Table II) that the velocity constants are higher in 62.0 per cent $CaCl_2$ than in any other mixture and that they decrease as the per cent of $CaCl_2$ decreases to 2.44 per cent. Thus in the case of K_M the value in 62.0 per cent $CaCl_2$ is 0.009, in 2.44 per cent $CaCl_2$ it is less by 0.00354, while in 15.0 per cent it is less by 0.0017, and in 35.0 per cent by 0.00041; if we multiply these numbers by the constant factor 0.251 they agree very closely with the figures for the increase in Na_4XCa. These values are plotted in Fig. 2, which shows that the decrease in K_M is directly proportional to the increase in the amount of Na_4XCa. Hence we assume that Na_4XCa acts as a negative catalyzer or inhibitor of the reaction $M \rightarrow B$.

An inspection of Table II shows that the value of K_A fluctuates with that of K_M except that as $CaCl_2$ increases the value of K_A rises more rapidly than that of K_M. This is also obvious from Fig. 1, which shows that the greater the per cent of $CaCl_2$ in the mixture the greater the maximum attained. Since this maximum increases as the value of $K_A \div K_M$ increases, it is evident that the value of $K_A \div K_M$ must rise as the per cent of $CaCl_2$ becomes greater. The value of $K_A \div K_M$ in the solution containing 1.41 per cent of $CaCl_2$ is 0.03333 while in the solution containing 62.0 per cent $CaCl_2$ it is 0.05889, an increase of 0.02556. If we calculate this increase for the other mixtures and plot the values so obtained against the per cent of $CaCl_2$ in the surface, we obtain a straight line as shown in Fig. 3. This indicates that $CaCl_2$ catalyzes the reaction $A \rightarrow M$; for if this were not the case the value of K_A and K_M would rise and fall in such a way that the value of $K_A \div K_M$ would remain constant.

It is evident from Figs. 2 and 3 that the values of K_A and K_M are determined by the amount of Na_4XCa and by the per cent of $CaCl_2$

in the mixture, and that when these values are experimentally determined for any two mixtures they can be calculated for any other mixture. When this is done we can calculate the course of the death curve in that mixture.

Having thus accounted for the death curves, we may turn our attention to the process of recovery. We find that, when tissue is removed from a mixture of NaCl and $CaCl_2$ and replaced in sea water, the resistance at once rises or falls and after a time becomes stationary. This rise or fall of resistance may be called recovery.

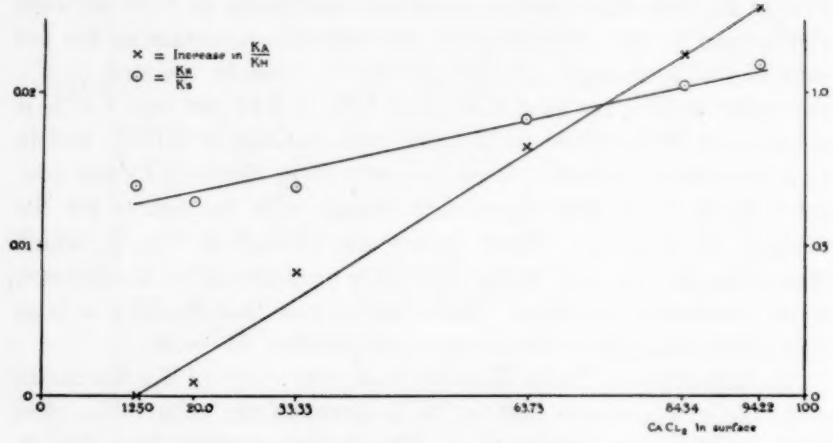

FIG. 3. Graph showing the increase of $K_A \div K_M$ and the value of $K_R \div K_S$ as the molecular per cent of $CaCl_2$ increases. The figure shows that $CaCl_2$ acts as a catalyzer of the reaction $A \rightarrow M$ (which has the velocity constant K_A) and also of the reaction $R \rightarrow S$ (which has the velocity constant K_R). The figures on the ordinate at the right show the values of $K_R \div K_S$; those on the ordinate at the left show the increase in the value of $K_A \div K_M$ over the value found in the mixture containing 1.41 per cent $CaCl_2$. The abscissæ denote molecular per cent of $CaCl_2$ in the surface (not in the solution).

In order to account for the facts we suppose that when we replace the tissue in sea water the reactions $O \rightarrow S \rightarrow A \rightarrow M \rightarrow B$ proceed at the rates which are normal for sea water. The manner in which the rate of recovery is calculated has been explained in detail in a previous paper.[1] It is assumed that during the exposure to any of the mixtures the following reactions occur: (1) $N \rightarrow O \rightarrow P$; (2) $R \rightarrow S \rightarrow T$; (3) $A \rightarrow M \rightarrow B$. By assuming values of the velocity

constants of these reactions we can approximate the observed results. The velocity constants thus found are given in Table II. An inspection of the table shows that all these velocity constants behave like K_A and K_M in that as the per cent of $CaCl_2$ in the mixture increases (beginning with 1.41 per cent $CaCl_2$) the value of the velocity constant first falls and then rises, and that this value in every case reaches its minimum in the mixture containing 97.56 NaCl + 2.44 $CaCl_2$. It would therefore appear that the reactions $N \rightarrow O \rightarrow P$ and $R \rightarrow S \rightarrow T$ are inhibited by Na_4XCa in the same manner as the reactions $A \rightarrow M \rightarrow B$. This is borne out by an inspection of Fig. 2. in which the decrease[7] of the velocity constants is plotted, together with the increase of Na_4XCa.

We have seen that the value of $K_A \div K_M$ increases as the per cent of $CaCl_2$ increases and we interpreted this to mean that the reaction $A \rightarrow M$ is catalyzed by $CaCl_2$. In the same manner we infer that the reaction $R \rightarrow S$ is catalyzed by $CaCl_2$, since we find that the value of $K_R \div K_S$ increases with increasing percentage of $CaCl_2$, as shown in Fig. 3. It is not certain that the curve does not reach a minimum in the mixture of 97.56 NaCl + 2.44 $CaCl_2$ but for practical purposes we may, for the present, regard it as a straight line.[8]

[7] By the decrease in the velocity constant is meant the decrease which we observe as we pass from the solution containing the highest per cent of calcium (38.0 per cent NaCl + 62.0 per cent $CaCl_2$) to mixtures containing smaller per cents of calcium. Thus the decrease of $K_M = 0.009 - K_M$; the decrease of $K_N = 0.00134 - K_N$; the decrease of $K_O = 0.0013266 - K_O$; and the decrease of $K_S = 0.00319 - K_S$. In the same manner we find that the increase in the amount of Na_4XCa = amount of $Na_4XCa - 0.000047$.

The decrease of the amount of K_A and K_R is not shown in the figure because it depends not only on Na_4XCa but also on the per cent of $CaCl_2$.

The fact that even in the presence of the maximum amount of Na_4XCa these velocity constants are greater than in sea water is of course to be attributed to the other substances present in sea water.

[8] Since in pure NaCl or $CaCl_2$ the salt compound Na_4XCa is not formed, we should expect that in these solutions all the reactions would be more rapid than in the mixtures. That this expectation is fully realized is evident from Table II.

The velocity constants are somewhat higher in NaCl than in $CaCl_2$; this is not explained by the assumptions already made but it does not seem desirable at present to make additional assumptions for this purpose. We might expect the values of $K_A \div K_M$ and $K_R \div K_S$ to reach a maximum in $CaCl_2$. This is actually the case. It might perhaps be expected that these values would fall to a minimum in NaCl. This is the case with $K_A \div K_M$ but not for $K_R \div K_S$.

The relation between K_N and K_O is taken as constant in the proportion of 100 to 99.

It is evident that when the constants have been empirically determined for two mixtures the constants for any other mixture can be calculated at once, since all of them depend in a definite manner on Na_4XCa (K_A and K_R also depend on the per cent of $CaCl_2$). The agreement between the constants thus obtained by calculation and those found by trial is fairly close, as is evident from Figs. 2 and 3.[9]

It has been shown in a previous paper that the height to which the recovery curve rises depends on the value of O: the value of O

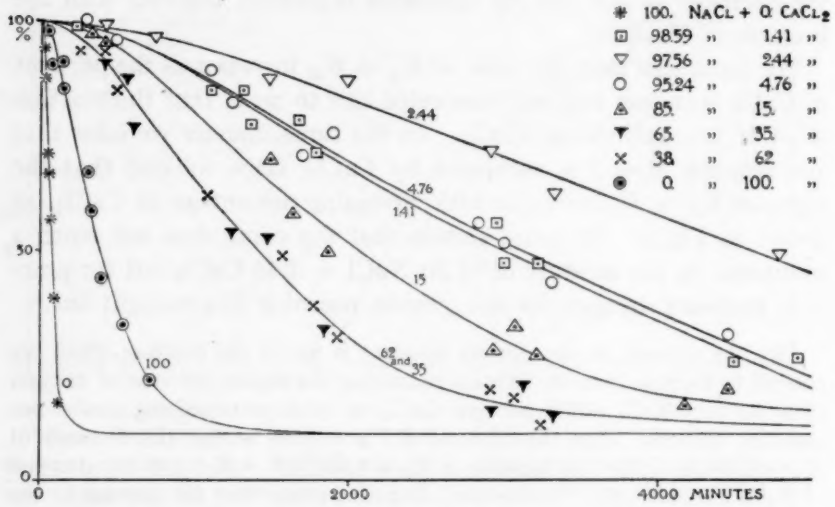

FIG. 4. Curves showing the value of $O + 10$ in 0.52 M NaCl, in 0.278 M $CaCl_2$, and in mixtures of these (the figures attached to the curves show the molecular per cent of $CaCl_2$ in the solution). The ordinates give the relative values of $O + 10$, the value in sea water being arbitrarily taken as 100 per cent. These values are obtained by exposing tissue to toxic solutions and then finding the level to which the resistance rises or falls after the tissue is replaced in sea water: they are therefore a measure of permanent injury. The abscissæ give the length of exposure to the toxic solution. The curves show the calculated values (using the velocity constants given in Table II). The points show the observed values; each represents the average of six or more experiments. Probable error of the mean less than 10 per cent of the mean.

[9] The constants obtained by calculation would fall exactly on the graphs in these figures while those found by trial are indicated by the points given.

+ 10 is shown for all the solutions[10] in Fig. 4, which shows the agreement between observation and calculation in respect to the final level reached by the recovery curve, but not in respect to speed of recovery, which depends more on the value of S than on that of O. The rate of recovery seems to be about the same in the mixtures as in the pure salts. In general it is found that the rise or fall is nine-tenths completed in about an hour.

Fig. 5 shows the calculated values of S; observed values are not given because they cannot be very precisely determined. This is

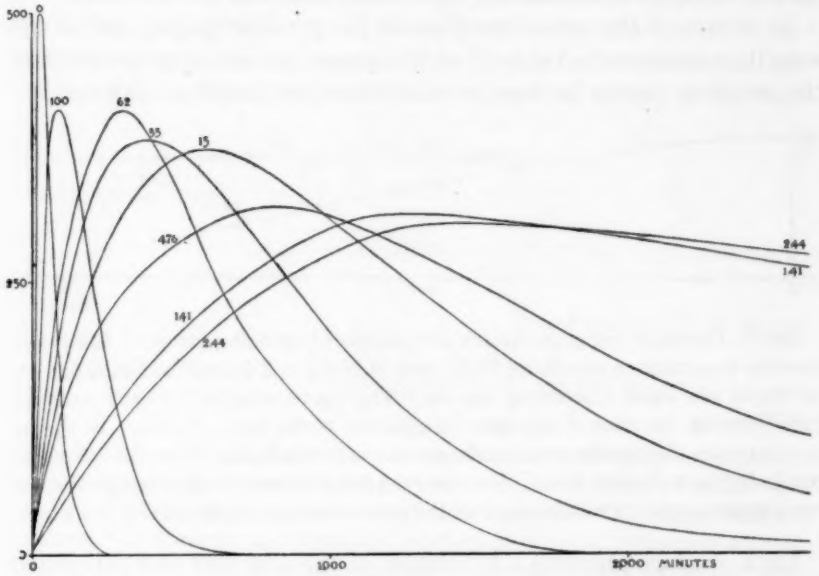

FIG. 5. Curves showing the (calculated) values of S in 0.52 M NaCl, 0.278 M $CaCl_2$, and in mixtures of these (the figures attached to the curves show the molecular strength of $CaCl_2$ in the solution). The curves show the values calculated from constants obtained by trial, which are given in Table II. The abscissæ represent the time of exposure to the toxic solution. The value of S at the start is in all cases 2.7.

[10] The values of $O + 10$ for solutions containing 2.44 and 15.0 per cent $CaCl_2$ differ slightly from those given in a former paper (Osterhout, W. J. V., *J. Gen. Physiol.*, 1920–21, iii, 15) for the reason that the curves here presented include a larger series of experiments. 10 is added to the value of O because the base line is taken as 10, just as in the case of M.

owing to the fact that S affects only the speed of recovery (not the final level attained) and as the speed is variable the most satisfactory procedure is to assume such values of K_R and K_S in the equation[11]

$$S = R\left(\frac{K_R}{K_S - K_R}\right)\left(e^{-K_R T} - e^{-K_S T}\right) + S_0\, e^{-K_S T}$$

as cause the closest approximation to the observed speed of recovery. The values of S thus obtained for each solution are shown in the figure. In general the speed of recovery, as calculated from these values of S, is in satisfactory agreement with the observations.

By means of the equations given in the previous paper, and of the velocity constants in Table II of this paper, we are able to calculate the recovery curves for any solution after any length of exposure.

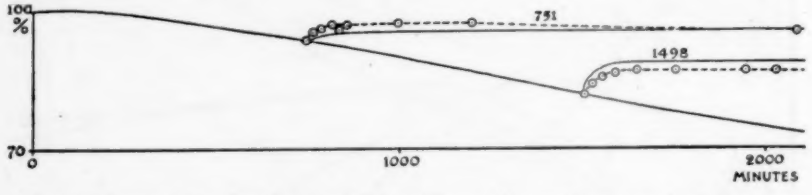

FIG. 6. Curves showing the electrical resistance (descending curve) of *Laminaria agardhii* in a mixture containing 97.56 mols of NaCl to 2.44 mols of $CaCl_2$ and recovery in sea water (ascending curves). The figure attached to each recovery curve denotes the time of exposure (in minutes) to the toxic solution. In the recovery curves the experimental results are shown by the broken lines, the calculated results by the unbroken lines. The observed points represent the average of six or more experiments. Probable error of the mean less than 10 per cent of the mean.

Lack of space prevents a tabulation of the observed and calculated values, but it is possible to exhibit graphically the data for three mixtures and for this purpose one in which recovery consists in a rise of resistance (Fig. 6), one in which it shows a moderate fall (Fig. 7), and one showing a very decided fall (Fig. 8) are presented. In general the agreement between observation and calculation is satisfactory for all the solutions employed in the investigation.

It might be thought that the number of constants is sufficient to make it possible to fit any sort of experimental curve and that the

[11] *Cf.* equation (3) of the preceding paper (Osterhout, W. J. V., *J. Gen. Physiol.*, 1920–21. iii. 145)

consequent agreement between observed and calculated results is less significant than would otherwise be the case. But, as a matter of fact, the constants are so related to each other and to the salt compound, Na_4XCa that the whole set of curves fits into a consistent scheme, so that when the constants are determined for any two mixtures the theoretical curves for all the other mixtures are thereby fixed. Under these circumstances the close agreement in the six different mixtures (ranging from 1.41 to 62.0 per cent $CaCl_2$) seems to be significant.

There seems to be no doubt that the behavior of the tissue is such as to indicate an underlying mechanism which is the same in all cases.[12] We have assumed that this mechanism consists in the production and decomposition of a substance, M, the amount of which, in the mix-

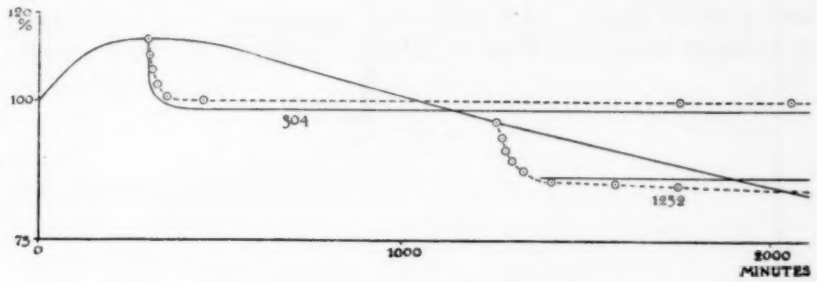

FIG. 7. Curves showing the electrical resistance (curve which ascends and descends) of *Laminaria agardhii* in a mixture containing 95.24 mols of NaCl to 4.76 mols of $CaCl_2$, and recovery in sea water (descending curves). The figure attached to each recovery curve denotes the time of exposure (in minutes) to the toxic solution.

In the recovery curves the experimental results are shown by broken lines, the calculated results by unbroken lines. The observed points represent the average of six or more experiments. Probable error of the mean less than 10 per cent of the mean.

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[12] This is shown, for example, by the fact that the rapidity of permanent injury (as observed after replacement in sea water) corresponds throughout with the rate of death, and that the rate of change of M corresponds throughout with the rate of change of O, S, and A. In other words if we change the solution in such a way as to increase (or decrease) the rate of one of the reactions on which the resistance depends we simultaneously increase (or decrease) the rates of all the others in a definite and predictable manner.

tures, depends largely on a compound Na_4XCa formed by the combination of Na and Ca with a constituent X of the protoplasm. It is not necessary to discuss these asumptions more fully at present. But it may be pointed out that two things seem to be fairly well established; (1) a consistent mechanism underlies the entire behavior of the

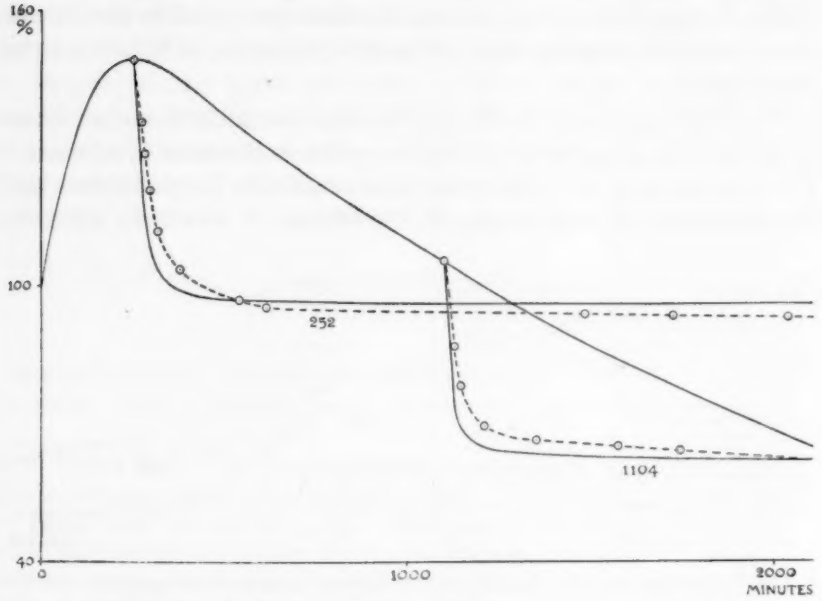

FIG. 8. Curves showing the electrical resistance (curve which ascends and descends) of *Laminaria agardhii* in a mixture containing 38 mols of NaCl to 62 mols of $CaCl_2$, and recovery in sea water (descending curves). The figure attached to each recovery curve denotes the time of exposure (in minutes) to the toxic solution. In the recovery curves, the experimental results are shown by the broken lines, the calculated results by the unbroken lines. The observed points represent the average of six or more experiments. Probable error of the mean less than 10 per cent of the mean.

tissue, and (2) its operation can be predicted with a fair degree of accuracy by means of the equations which have been developed. The predictive value of these equations may be regarded as permanently established, since it does not depend on our views regarding the underlying assumptions.

SUMMARY.

1. The equations which serve to predict the injury of tissue in 0.52 M NaCl and in 0.278 M $CaCl_2$ and its subsequent recovery (when it is replaced in sea water) also enable us to predict the behavior of tissue in mixtures of these solutions, as well as its recovery in sea water after exposure to mixtures.

2. The reactions which are assumed in order to account for the behavior of the tissue proceed as if they were inhibited by a salt compound formed by the union of NaCl and $CaCl_2$ with some constituent of the protoplasm (certain of these reactions are accelerated by $CaCl_2$).

3. In this and preceding papers a quantitative theory is developed in order to explain: (*a*) the toxicity of NaCl and $CaCl_2$; (*b*) the antagonism between these substances; (*c*) the fact that recovery (in sea water) may be partial or complete, depending on the length of exposure to the toxic solution.

THE RATE OF OVULATION IN THE DOMESTIC FOWL DURING THE PULLET YEAR.

By SAMUEL BRODY.

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It is well known among poultrymen that ovulation in the hen is an orderly process with a fairly predictable average production of eggs for each month of the year. The object of this paper is to contribute an idea toward the formulation of an hypothesis concerning the principles underlying this orderly process of ovulation. This idea is based on a suggestion of Loeb[1] that growth, or at any rate the limiting factor of growth, is in the nature of an autocatalytic monomolecular reaction. The rate of ovulation may reasonably be taken as an index of growth of the eggs, and if the limiting factor of growth of eggs is an autocatalytic reaction, then the rate of ovulation should be expressible by the equation of such a reaction.

The application of this equation to experimental data is familiar to the chemist.[2] Robertson[3] and Ostwald[4] were the first to apply the equation to the phenomena of growth. In the formulation of the equation it is assumed that the velocity of reaction at any moment is proportional to the quantity of the reacting substance $(A - x)$ at that moment, and also to the quantity of the product of the reaction

[1] Loeb, J., *Biochem. Z.*, 1907, ii, 34; *7th Internat. Zool. Cong.*, 1907; The dynamics of living matter, New York, 1906; *Univ. California Pub., Physiol.*, 1905–10, iii, 61.

[2] Ostwald, W., Lehrbuch der allgemeinen Chemie, Leipsic, 1903, i. Lewis, G. N., *Z. physikal.Chem.*, 1905, lii, 310; *Dept. Interior, Bureau Gov. Labor., Chem. Lab., No. 30*, 1905.

[3] Robertson, T. B., *Arch. Entwcklngsmechn. Organ.*, 1908, xxv, 581; *Am. J. Physiol.*, 1915, xxxvii, 1, 74; Principles of biochemistry, for students of medicine, agriculture and related sciences, Philadelphia, 1920.

[4] Ostwald, W., Vorträge und Aufsätze über Entwicklungsmechanik Organismen, Leipsic, 1908, v.

x, present at that moment, which acts as the catalyst of the reaction; that is

$$\frac{dx}{dt} = K_1 x\,(A - x) \qquad (1)$$

where $\frac{dx}{dt}$ stands for the momentary velocity or rate of reaction, and K_1 is the velocity constant. Equation (1) cannot be applied to experimental data since the momentary velocity of the reaction is not known, and since the velocity is not expressed as a function of time. It is therefore first integrated obtaining

$$\log \frac{x}{A - x} = K_1 A t + C \qquad (2)$$

where C is the integration constant. The value of the integration constant C is found by an analysis of the meaning of t in equation (2) above. Equation (1) represents a curve of a rising and falling type, the maximum or turning point occurring when $x = A - x$; that is, when the reaction is half way completed, and when the reaction is at the maximum velocity. At that point, therefore, $\log \frac{x}{A - x} = 0$. It is most convenient to count the time from this maximum or turning point, that is t at this point is just equal to zero; therefore, $K_1 A t = 0$ and also $C = 0$; if it is agreed to count time from the maximum velocity, then $C = 0$, and equation (2) becomes

$$\log \frac{x}{A - x} = K_1 A t \qquad (3)$$

t in equation (3) is then the time on either side of the maximum point, counted from that point as zero. Equation (3) may be more conveniently written

$$\log \frac{x}{A - x} = K_1 A\,(t - t_1) \qquad (4)$$

where t_1 is the time from the beginning of the reaction to the maximum point; t is any time from the beginning of the reaction chosen for discussion; and $(t - t_1)$ is therefore the difference of time from the maximum point to the chosen time t. The minus sign between t and t_1 indicates difference in time between the maximum and

any chosen time t, anywhere along the reaction curve, on either side of the maximum, rather than that t_1 is negative. Equation (4) may be still further simplified by writing it

$$\log \frac{x}{A - x} = K (t - t_1) \quad (5)$$

where K is written for $K_1 A$. Equation (5), used by Robertson[3] in the study of growth, may now be applied to the study of data on ovulation.

A large amount of data on ovulation of the domestic fowl is found in the work of the several Agricultural Experiment stations in this country. The best known published records are undoubtedly those prepared by Pearl[5] on the weighted mean monthly egg production of barred Plymouth Rock pullets representing 4,210 birds covering the records kept from 1899 to 1907 at the Maine Agricultural Station, and the results of the international egg laying contests conducted by the Storrs Experiment Station.[6] The average monthly production of 1,000 White Leghorn pullets during the seventh international contest[6] will be taken as the second example for computation.

It might be well before applying equation (5) to these data to redefine them in terms of ovulation during the pullet year. x = number of eggs laid from November 1 of the pullet year up to the end of any month, t. A = total number of eggs laid in the *natural laying season* which lasts very nearly 1 year, November 1 of pullet year to November 1 of the succeeding year. Some eggs may be, and in fact are, laid outside this arbitrarily defined limit of 1 year. However, all published records adhere to this arbitrary year, and hence A is tentatively defined as eggs laid from November 1 to November 1 of the succeeding year. t_1 = time in month when $x = A - x$ = time required to lay half of the total number of eggs laid in 1 year = time when the monthly rate of laying is at its maximum. K = velocity constant found by substituting values for x, A, t, and t_1, and solving for K. Substituting the values of A, K, t, and t_1, and solving for x, we obtain the following calculated values for the two examples chosen,

[5] Pearl, R., *U. S. Bureau Animal Industry, Bull. 110*, 1910, pt. 2.

[6] Card, L. E., and Kirkpatrick, W. F., *Storrs Agric. Exp. Station, Bull. 100*, 1919, pt. 2, 35.

which are tabulated with the experimental values. Robertson's tables[7] are helpful in solving for x, the calculated number of eggs laid.

Month.	No. of month. t	Barred Plymouth Rock.		White Leghorn.	
		Experimental values.	Calculated values.	Experimental values.	Calculated values.
Nov.	1	4.63	10.8	6.5	7.3
Dec.	2	13.54	16.8	13.1	12.2
Jan.	3	25.25	25.2	20.2	19.9
Feb.	4	36.12	36.7	30.7	31.5
Mar.	5	52.23	50.6	48.4	47.5
Apr.	6	68.08	66.1	66.9	67.5
May.	7	82.00	81.5	89.5	89.5
June.	8	94.46	95.0	109.2	110.1
July.	9	105.53	105.8	127.2	127.5
Aug.	10	115.17	113.7	143.8	140.2
Sept.	11	123.36	119.2	156.8	149.0
Oct.	12	128.86	123.0	162.8	154.8

Similar results are obtained with the other principal breeds of birds, the equation for Plymouth Rocks being

$$\log \frac{x}{129 - x} = 0.212\ (t - 5.9)$$

and the equation for White Leghorns

$$\log \frac{x}{163 - x} = 0.235\ (t - 6.64)$$

In the equation for White Leghorns, 163 is the value of A since the total number of eggs laid in the year is in round numbers 163 eggs. It takes 6.64 months to lay half this number of eggs. 0.235 is the constant of the reaction.

It is clear from the tabulation that the agreement between the experimental and calculated values of x (eggs laid) is very good for three-quarters of the laying season—January to August. On the other hand the per cent deviation in the first 2 months (November and December) and the last 2 months (September and October) is very great. The deviations in the last 2 months (September and

[7] Robertson, T. B., *Univ. California Pub., Physiol.*, 1910–15, iv, 211.

October) can be easily corrected by assuming the value of A to be slightly larger than the recorded number of eggs laid during the arbitrarily defined laying year of November 1 to the following November 1. Thus, instead of taking the value of A to be 163 eggs for the

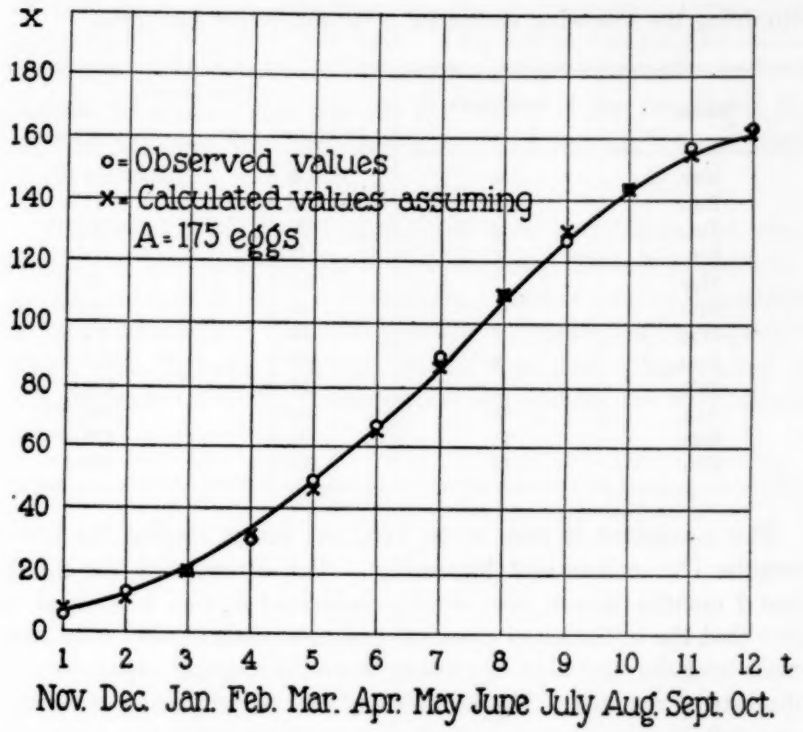

FIG. 1. Curve showing the average number of eggs laid by the White Leghorn fowl at the Storrs Seventh International Egg Laying Contest. Ordinates represent the number of eggs laid (x) for the time t; abscissæ represent the time t from Nov. 1, the beginning of the observation, to any time of the year.

White Leghorns, 175 eggs are taken, an increase of only 12 eggs over the recorded value, a number which will probably be laid by this breed outside the arbitrary, conventional year; then an excellent agreement is obtained as shown in the following tabulation and in Fig. 1.

Assume then $A = 175$; t_1 is shifted to 7 months and the equation becomes

$$\log \frac{x}{175 - x} = 0.222\ (t - 7)$$

obtaining the following values for x for the White Leghorns.

Month.	No. of month. t	White Leghorn.	
		Experimental value.	Calculated value.
Nov.	1	6.5	7.9
Dec.	2	13.1	12.6
Jan.	3	20.2	20.1
Feb.	4	30.7	31.0
Mar.	5	48.4	46.4
Apr.	6	66.9	65.6
May.	7	89.5	87.5
June.	8	109.2	109.2
July.	9	127.2	129.0
Aug.	10	143.8	144.0
Sept.	11	156.8	155.0
Oct.	12	162.8	162.4

The agreement is seen to be excellent except during the first 2 months (November and December). The discrepancy during the first 2 months cannot, however, be considered serious, in view of the fact that the initiation of chemical processes even *in vitro* is in many cases irregular and does not follow the mathematical expressions for the rate of reaction. The initiation of processes in living organisms, especially in the case of ovulation, may be attended in addition by purely mechanical difficulties and irregularities. The discrepancy might also be explained on evolutionary grounds following the argument of Pearl.[5] Pearl calls attention to the fact that the wild *Gallus*, the ancestor of the domestic fowl, does not lay during the winter months, and that the winter laying period is not a part of the natural or normal reproductive cycle of the hen. The high variability of egg production during this period is explained by Pearl on this basis.

SUMMARY.

The rate of ovulation of the domestic fowl may be expressed by the equation of an autocatalytic chemical reaction. This is not surprising in view of the fact that the rate of growth may also be expressed by such an equation, and that the rate of ovulation is probably an index of the growth of the eggs. This brings the phenomenon of ovulation in the hen under the general subject of growth, and substantiates the generality and the probability of the hypothesis that growth, or at any rate the limiting factor of growth, is an autocatalytic reaction.

The idea of applying the equation of growth to the rate of ovulation in the fowl suggested itself while studying the rate of growth of the dairy cow with Mr. A. C. Ragsdale, Chairman of the Department of Dairy Husbandry. I take pleasure in expressing my appreciation to Mr. Ragsdale, and Mr. H. L. Kempster, Chairman of the Department of Poultry Husbandry in this station, for their encouragement.

THE PHAGOCYTOSIS OF SOLID PARTICLES.

I. Quartz.

By WALLACE O. FENN.

(*From the Laboratory of Applied Physiology, Harvard Medical School, Boston.*)

(Received for publication, January 15, 1921.)

Measurement of Phagocytosis.

The usual procedure for measuring phagocytosis is to incubate suspensions of leucocytes and solid particles or bacteria together in a test-tube for a given length of time; then to remove a sample and make a smear of the mixture on a slide which is stained, mounted, and counted at leisure. When bacteria are used as objects for ingestion, as in opsonic index determinations, the actual number of bacteria inside a given number of leucocytes is counted and comparisons are made on the basis of the average number of bacteria taken up per leucocyte per unit of time. It is impossible to do this with solid particles which are usually larger than bacteria and hence may almost completely fill the cell in a short time or become easily superimposed.

For this reason Hamburger (1), the author of the only extensive quantitative experiments on phagocytosis of solid particles, always counted the per cent of leucocytes containing solid particles per unit of time. On theoretical grounds this method possesses the objection pointed out by McKendrick (2) that it is not a measure of the amount of work done but a measure of the number of cells which have done the work. He shows, however, that if a normal frequency curve for the distribution of the bacteria in the leucocytes is assumed, the number of bacteria per leucocyte, *i.e.* the amount of work done, can be calculated from the per cent of empty leucocytes, the former being a logarithmic function of the latter, and he suggests that the determination of opsonic index may be simplified by counting only the empty leucocytes.

Madsen and Watabiki (3) have avoided this difficulty, in measurements of the rate of phagocytosis of bacteria at different temperatures, by measuring the time curves of the number of bacteria taken up per leucocyte at each temperature. By certain assumptions they succeeded in fitting their experimental curves to the formulas for monomolecular or bimolecular reactions, and thus determining a constant, K, which represented the rate of the reaction. There seemed to be in this case, however, no particular significance to the obedience of the curves to the laws for chemical reactions.

In the experiments on phagocytosis of solid particles to be described in this paper it was found possible to avoid the objections in former methods and to analyze the reactions quantitatively in terms of the number of collisions occurring between cells and particles. To accomplish this, particles of carbon or quartz of uniform size were incubated with leucocytes. At frequent intervals a sample was removed to an ordinary blood-counting chamber and the *number of particles not taken up* by the leucocytes was counted.

If the number of cells present is large and remains constant throughout the experiment, the number of collisions varies only with the number of particles, and it should be possible to calculate the rate of phagocytosis, K, from the equation for a monomolecular reaction, $K = \frac{1}{t} \log \frac{A}{A - x}$, where A is the number of particles originally present and x is the number ingested by the leucocytes in the time, t. The fact that K is usually found to be constant shows that we are dealing with a process like a monomolecular reaction in which the collisions can be watched under the microscope. The leucocytes can take up so many particles that their capacity does not diminish sufficiently to limit the rate of the reaction. Certain uncontrollable complicating factors which cause K to vary will be discussed with the experimental results and in a subsequent paper.

A constant K, calculated in this way, means that the same per cent of the particles present is being ingested per unit of time; *i.e.*, K is independent of the actual number of particles present. In other words, it is *not the number of collisions but the chances of collision which determine* K, other things being equal. Since the chances of collision

depend upon the size and density of the particles in relation to the size and density of the cells, large particles should be taken up more rapidly than small particles. This was found to be true, and, moreover, a direct proportionality was found to exist between the experimental constant, K, and the relative chance of collisions as calculated. It will be seen that this method presents great difficulties as a means of comparing the rate of phagocytosis of different particles and it was finally discarded in favor of a simpler method which will be described in a subsequent paper. The results by the first method possess considerable theoretical interest, however, and the consideration of the chances of collision should be of interest perhaps in opsonic index work where this factor has never been taken into consideration.

The technique of handling the quartz suspensions will first be described; then the method of calculating the chances of collision; and the experimental results, using three different sizes of quartz particles. Similar experiments with carbon and comparisons between quartz and carbon will be described in later papers.

The Suspensions.

The first requirement for these experiments is to have the particles of as nearly uniform size as possible. Suspensions prepared by Hamburger's method would not be sufficiently uniform to admit of a calculation of the chances of collision. Uniformity can be obtained by centrifugalization or settling. The former is quicker but the product is not so good nor so easily controlled. In both methods the principle consists in washing out the particles which are too small. The larger particles may be left in the suspension until a uniform sample is needed, when the suspension is shaken up and the large ones are either centrifugalized out or allowed to settle out by gravity. In the centrifuge, however, it is quite impossible not to set up currents which carry some excessively large particles to the top. The small particles can, however, be removed in the centrifuge and the sediment can thus be resuspended and allowed to settle out by gravity. It settles with a sharp line at the top from which a uniform sample can be withdrawn.

This combined method was the one usually used in these experiments. Suspensions of this sort of carbon and quartz have been

kept for nearly a year at room temperature, without sterilizing, and there has always been an abundant supply of uniform particles in each when shaken up and allowed to settle. *Uniform* suspensions cannot be kept, however, without the addition of acacia or some other protective colloid to prevent agglutination.

Chances of Collision.

If a suspension of cells of diameter C and velocity (under the influence of gravity) Vc, and of particles of diameter P and velocity Vp, is allowed to settle in a test-tube, the chances of collision, R, between them will be proportional to the velocity of the particle relative to the leucocyte and to the square of the sum of their diameters or

$$R = (Vp - Vc)\ (C + P)^2 \qquad (1)$$

The last factor is derived by a consideration of Fig. 1. If an infinitely small particle is settling down, its chance of hitting a single cell C is proportional to the cross-sectional area of C or πC^2. If, however, the particle has a finite diameter P, its center may miss the edge of the cell by a distance $\frac{P}{2}$ and still collide. The effective cross-sectional area of C as a target for a particle is thus increased to $\pi(C + P)^2$. The chance that P will hit C is, therefore, proportional to $(C + P)^2$.[1]

In these experiments, however, the suspensions were not allowed to settle out in stationary test-tubes as the formula could no longer be applied to cells and particles which were resting on the bottom. Instead, the test-tubes holding 1 to 2 cc. of the mixture were placed horizontally on a drum revolving slowly about a horizontal axis. It can be shown, however, that the same formula applies to this case. Consider first a case where there is no air bubble in the tube. Each cell and particle in the mixture is settling at a constant rate in a uniformly rotating medium, and at the end of one revolution will have returned exactly to the original position in the tube, having

[1] The writer is indebted to Dr. E. K. Carver, National Research Fellow in Chemistry, of the Wolcott Gibbs Memorial Laboratory of Harvard University, for assistance with this formula.

described a circle the circumference of which is equal to the distance which each cell or particle would have settled during the time of one revolution either in a straight line or otherwise.

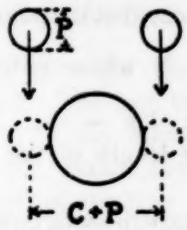

FIG. 1. Diagram illustrating chance of collision between a particle, P, settling down toward a cell, C.

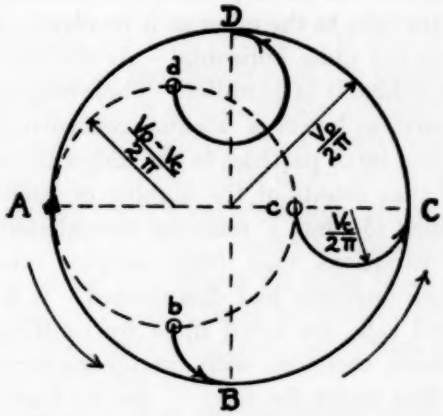

FIG. 2. Diagram illustrating orbits described by a particle at A and cells at b, c, and d in a rotating medium. The figure represents a cross-section of the medium which is considered to be rotating clockwise. The relative direction of rotation of particles and cells settling by gravity is, therefore, counter-clockwise, as indicated by the arrows. Collisions occur at B, C, and D. Chance of collision is proportional to the circumference, $Abcd$.

The circumference thus equals $\frac{V}{n}$, where n is the number of revolutions per unit of time and V the velocity. Let the circle $ABCD$ (Fig. 2) be the orbit described by a particle, P, starting at A in the direction

of the arrow. Its radius equals $\frac{Vp}{2\pi}$.[2] Then the cells of velocity Vc which will collide with the particle at B, C, and D after one-quarter, one-half, and three-quarters of a revolution, respectively, must have been originally located at the points b, c, and d. But all these points lie on a circle (Fig. 2) whose radius is $\frac{Vp}{2\pi} - \frac{Vc}{2\pi}$, and whose circumference is therefore, $Vp - Vc$. Obviously the chance of collision depends upon the length of the line upon which colliding cells may lie.

In some of the following experiments, however, these ideal conditions were not quite realized, especially at the beginning before the theory of the chances of collision had been thoroughly worked out. Instead, an air bubble was allowed to be present and in many cases traveled from one end of the tube to the other as it revolved, owing to the fact that the tube was not quite horizontal. As the bubble passes along the tube it leaves behind it little eddies. These may be seen in a thick suspension of quartz as layers of unequal concentration of particles caused by the piling up of particles in the eddies by centrifugal force. It was observed that counts of the number of particles from small amounts of samples (5 c.mm.), removed immediately after stirring, showed greater variations than from samples taken when these inequalities in concentration had disappeared. It is probable that both particles and cells are acted upon by centrifugal force when stirred up, and move, therefore, with exactly the same *relative* velocities as when settling under the force of gravity alone. However the mixture is stirred, there would be no collisions if all particles and cells had exactly the same velocities, for they would merely be carried along by the current. Any collisions, then, must be due to differences in velocities.

In the smallest of these particles brownian motion was just perceptible. Even if the particles were so small, however, as to have no velocity under gravity and therefore very active brownian motion, this fact would not affect the calculation of the chances of collision with a cell which is literally "sweeping up" the particles with a velocity

[2] n is taken equal to 1.

of 1.67 cm. per hour, since the number of particles which got in front of the cell by their brownian motion would be just balanced by the number which would get out of the way of the cell.

Verification of the Theory by Varying the Speed of Rotation.

According to the theory, then, the chances of collision determine K, other things being equal. The fact that the chance of collision is proportional to $Vp - Vc$, *i.e.* the difference between the speeds of settling of particles and cells, makes possible a verification of the theory by varying the speed of rotation of the tubes in which the phagocytic mixtures are incubated.

Returning to Fig. 2, it is evident that the orbits described by the particles, being equal to $\frac{V}{n}$, must decrease with an increase in n, the number of revolutions per unit of time. It should be possible by increasing n to decrease the diameter of the orbits nearly to zero. Under such conditions there should be less phagocytosis than when the mixture is rotated more slowly. In a slowly rotated mixture the particles have plenty of time to settle down to the neighboring cells. To test this prediction under ideal conditions, six small tubes, 3 mm. inside diameter and 1.5 cm. long, sealed at one end, were prepared. At the beginning of the experiment all these tubes were filled with the mixture of cells and quartz particles immediately after the cells were added. The open ends of the tubes were then sealed by dipping in melted paraffin, excluding all air.

Three of the tubes were then put on a drum rotating at 0.3 revolution per minute and three on one rotating at 19 revolutions per minute. Counts were then made of the number of particles present in the remainder of the original mixture. At intervals of about 1 hour one tube was taken from each drum and the number of particles not yet ingested was counted. From these data the phagocytosis constant, K, was calculated by the equation for a monomolecular reaction.

As predicted, the particles are ingested nearly two and one-half times as rapidly in the slowly revolving tube. Likewise, the cells aggregate more rapidly in the slow tube, due to the greater number of collisions between them.

The result was as follows:

Hrs.	19 revolutions per minute.		0.3 revolution per minute.	
	No. of particles.	No. of cells.	No. of particles.	No. of cells.
0	324	76	324	76
0.6	262	76	124	48
1.75	157		55	
2.6	64	53	9	32
	$K = 0.22$		$K = 0.52$	

(Counts refer to volumes of 0.02 mm.^{3})

The diameter of the orbits of cells and particles has been calculated from the equation Diameter $= \frac{V}{\pi n}$, and is compared in the following tabulation with the average distance between cells and particles calculated from the equation

$$\text{Average distance} = \sqrt[3]{\frac{\text{Total volume of suspension}}{\text{No. of cells} + \text{No. of particles}}} = 37\mu$$

	Cells. μ	Particles. μ
Average diameter	9.0	2.9
Diameter of orbit at 19 revolutions per min.	4.6	7.3
" " " " 0.3 revolution " "	279	462

These results show that the diameters of the orbits of cells and particles at 19 revolutions per minute are small compared to the distances between them. It is, therefore, only due to the fact that the laws of chance prevent all particles from being equidistant from each other (*i.e.* 37μ) that there was any phagocytosis at all at 19 revolutions per minute. In this case, also, when the cells are practically stationary, brownian motion probably plays some part in bringing particles to the cells. Centrifugal force is another factor which would have a very slight effect in causing collisions. It was calculated that at 19 revolutions per minute 12 per cent of the particles and 8 per cent of the cells would be centrifugalized against the wall of the tube and that it would take 21 minutes for a particle to overtake a cell 37μ distant,

both moving under centrifugal force alone. Centrifugal force is not, therefore, an appreciable factor except in the case of those cells which become "plastered" against the wall of the tube and there ingest particles. This could have been avoided by rearranging the tubes on the drum at intervals.

This experiment was done under ideal conditions, however, in that there was no air bubble present in the tube. When such a bubble is included in a tube which is slightly inclined to the horizontal, it travels up and down in the tube and stirs the mixture. Under such conditions the rate of phagocytosis, K, should vary in direct proportion to the speed of rotation. This also is true. Thus K was increased from 0.17 to 0.76 (4.5 times) by an increase from 7 to 27 revolutions per minute (3.9 times).

These two experiments offered a very satisfactory proof of the theory at the outset and the following predictions may be made with considerable assurance.

1. The rate of phagocytosis, K, may be calculated from the equation for a monomolecular reaction and will remain constant in any experiment as long as the concentration of cells remains constant.

2. For a given leucocyte suspension in a given medium, K is determined by the chances of collision between cells and particles.

3. The chances of collision vary with the method of stirring (*i.e.* speed of rotation, etc.) but the ***relative*** chances of collision, R, are applicable however the cells and particles are kept in suspension.

4. The relative chance of collision of any particle is given by the formula $R = (C + P)^2 (Vp - Vc)$.

5. The equation $\frac{K_1}{K_2} = \frac{R_1}{R_2}$ should be true for any two sizes of particles, 1 and 2.

Velocity of Particles.

The relative chances of collision are, therefore, very simply calculated if the diameter and velocity of the particles and cells are known. The simplest way of determining the velocity of the particles is by direct observation of the speed with which the suspensions settle out in the centrifuge bottle. This can only be done accurately in a suspension of particles of such a uniform size that they all settle at

the same rate leaving a clear liquid above. In all the suspensions used in these experiments this was possible. From the velocity so obtained, the diameter can be calculated by Stokes's law.

For example, if a suspension has been allowed to settle for 1 hour until 5 cm. of clear water remain at the top, the upturned capillary tip of a siphon is now placed 7 cm. below the surface, and the upper 2 cm. of the suspension are drawn off, the velocity of these particles is somewhere between 5 and 7 cm. per hour. It is probably given most accurately by the formula

$$V_{av} = V_g - \frac{V_g - V_s}{\sqrt{2}} \qquad (2)$$

where V_s and V_g are the smallest and largest velocities, respectively. This may be seen from a consideration of Fig. 3. Here the abscissæ represent velocities. Ordinates represent the number of particles of

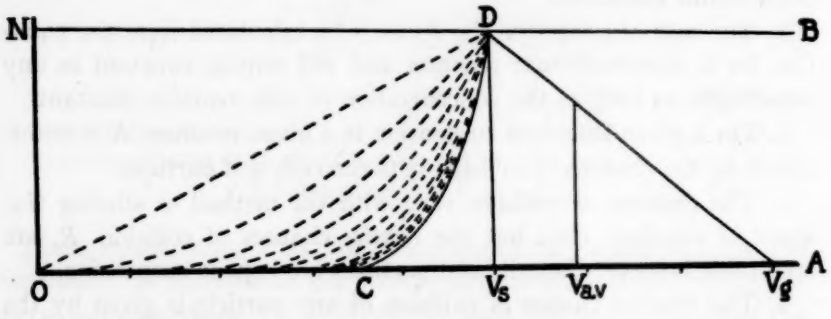

FIG. 3. Ordinates represent number of particles, abscissæ represent velocities or diameters squared. Original raw material is represented by *AONB*, *N* particles of each velocity being assumed. Dotted lines show portions removed after ten successive settlings. *ACDB* is the permanent suspension before large particles are removed. V_gCD is, then, the frequency curve of a uniform suspension removed from the top as suspension *ACDB* settles. V_{av} is the average velocity.

each velocity originally present; it is assumed that there were the same number, *N*, of each in the original raw material. The area, *AONB*, then represents the original suspension. The dotted lines *DO*, etc., outline the parts of the suspension which were successively discarded with the supernatant liquid after being allowed to settle ten times until particles of velocity, V_s, had just settled out. 50

per cent of the particles, which settle only half this distance, are removed each time.

The suspension as kept permanently is then represented by the area $ACDB$. To prepare a uniform suspension the whole is then shaken up, allowed to settle, and the upper layer of the suspension pipetted off at such a point that only particles of velocity smaller than V_g are obtained. The final suspension is then represented by the area V_gCD, and the average velocity must be measured by the perpendicular bisector of this area. Since V_sCD is small compared to the whole it may be neglected, particularly as V_s is necessarily more or less indeterminate in practice. The average V is, then, best given by the bisector of the triangle V_gV_sD or V_{av} which can be shown by elementary geometry to have the value given in equation (2) above.

Stokes's law (4) gives the velocity, V, in centimeters per second of a spherical body of radius, r, and density, D, in a medium of absolute viscosity, η, and density, d, as

$$V = \frac{2\,(D - d)}{9\,\eta}\, gr^2$$

whence

$$r = \sqrt{\frac{9\,V\eta}{2g\,(D - d)}}$$

Introducing values $\frac{0.010 \text{ erg second}}{\text{centimeter}^3}$ for η (5) and 981 $\frac{\text{centimeter}}{\text{second}^2}$ for g, and changing V into velocity in $\frac{\text{centimeter}}{\text{hour}}$ and r from centimeters to microns we have

$$r = \sqrt{\frac{4.5\ V\ 0.01 \times 10^8}{981\ (D - d)\ 3{,}600}}$$

and

$$\text{Diameter} = \sqrt{\frac{5.1\ V}{(D - d)}} \qquad (3)$$

For the purposes of these experiments it is possible to use this formula directly without allowing for the differences in the density and viscosity of the phagocytic mixtures. The viscosity has no effect on the relative chances of collision because it modifies the

velocity of both the cells and particles equally. Since the density of the medium was only 1.01 this can also be disregarded as small in comparison with the density of the particles, 1.81 (carbon) and 2.68 (quartz). As far as the leucocytes were concerned with a density 1.11, this was really allowed for largely by the necessity of measuring their velocity in a medium of sodium chloride of density 1.007.

In the case of suspensions which are not uniform enough to settle with a sharp boundary[3] it is impossible to determine anything but the velocity of their smallest particles by direct observation. For such cases a method was devised which is known as the "stop-cock" method. It was also necessary to use this method for determining the velocity of the leucocytes. The method consists in allowing the suspension to settle out in a glass tube provided with a specially made stop-cock, such that there was no constriction in the inside diameter; there was, thus, a straight uniform tube all the way through the stop-cock. After a given interval of time the stop-cock is turned. The change of concentration in the suspension above the stop-cock is determined by counts of the numbers of particles present before and after settling. If there are h centimeters of suspension above the stop-cock and if the concentration before and after is given by C and C_1, the velocity is given by the formula

$$V = \frac{h\,(C - C_1)}{Ct} \qquad (4)$$

This method gives an average figure for the velocity because the small particles which settle too slowly compensate for the large ones, all of which may have passed below the stop-cock before it is turned. Obviously, however, the diameter which is calculated from the average velocity is not the average diameter because the velocity is proportional to the square of the diameter. The agreement between results by this method and measurements of the diameter of particles by microscopic methods was, however, sufficiently good for these purposes as shown in Table I. The agreement is in itself proof of the uniformity of the suspensions used.

In the table are also included some figures for the diameters of these suspensions by the method of evaporating to dryness a suspension containing a known number of particles, and calculating the

[3] This is the case with suspensions from which the large particles were removed by centrifuge instead of by settling.

diameters on the assumption that each particle is a sphere. It is doubtless due to the irregular sizes of the particles that this method gives results which are uniformly lower than the other methods. At the end of the table are also included some figures for the smallest diameters of the particles as calculated from the velocity with which the upper boundary of the suspension settles under gravity; *i.e.*, the velocity of the smallest particles. This figure can readily be obtained from the stop-cock method in addition to the figure for the

TABLE I.

Measurements of Diameters in Microns of Three Sizes of Quartz Particles by Various Methods.

Method.	Diameters.		
	Size 1.	Size 2.	Size 3.
	μ	μ	μ
Dry weight	3.55	3.2	
	3.46	3.14	2.16
Microscopic	5.01	4.34	2.32, 2.44
	4.63 ± 0.05	4.08 ± 0.05	2.93 ± 0.036
Stop-cock method: Counting	4.67	4.44	2.82
	4.98	4.10	2.62
Stop-cock method: Colorimeter	5.05 ± 0.14	4.05 ± 0.08	2.7 ± 0.04
Smallest diameter	3.04	2.65	1.93
	3.26		2.16

average velocity and gives a good idea of the degree of uniformity of the suspension. To avoid counting, the stop-cock method was usually modified by the use of the colorimeter for comparing the concentration of the suspension before and after settling.[4] All the measurements in Table I were made on suspensions prepared separately

[4] For this purpose underneath illumination only was used so that the intensity of the transmitted, not the diffracted, beam was measured. The use of the colorimeter is perhaps a doubtful measure on theoretical grounds unless the suspensions are thoroughly uniform, because it assumes that the amount of light transmitted is proportional to the number of particles without respect to size. After settling there are of course relatively fewer large particles.

from the same original stock bottle, the large particles being removed by centrifugalization each time. It is not surprising, therefore, that the measurements should differ somewhat at different times even by the same method.

Diameter and Velocity of Leucocytes.

The leucocytes were obtained from rats. The animals were injected intraperitoneally with a suspension of aleuronat, and the peritoneal cavity was opened on the following day and washed out with 0.5 per cent sodium citrate in 0.9 per cent sodium chloride solution. The cells were then centrifuged once to get rid of the citrate and resuspended in salt solution. If the cells are not washed thus it is necessary to have so much citrate present to prevent clotting of the exudate that it prevents phagocytosis.

For these experiments it was of first importance to determine the diameter and velocity of the leucocytes. These determinations had to be done independently as the density could not be measured directly. The diameter was measured directly in the microscope on fresh specimens before they had had time to spread on the microscope slide. The velocity was determined by the stop-cock method. An average of five determinations was 1.67 ± 0.05 cm. per hour. The figures were as follows: 1.63, 1.56, 1.83, 1.47, 1.84.

Since, however, the leucocytes are not of the same size it is necessary, in order to calculate the chances of collision with any accuracy, to divide the cells into three groups of three average diameters and calculate the chances of collision for each group separately. These calculations were made as follows: The diameters, D, of the leucocytes were measured as already described. From these data a frequency curve was obtained which was divided into three parts (Table II). The relative velocities of the three groups were then calculated by the formula $V = D^2K$, where K is a constant. From these velocities the average velocity may be calculated as shown by multiplying the velocity for each group by the percentage of total cells in that group and dividing the sum of the results by 100. But the average velocity was found by experiment by the stop-cock method to be 1.67 ± 0.05. Equating these we have

$$1.67 \pm 0.05 = 81.0\,K$$
$$K = 0.0206 \pm 0.00062$$

Substituting this value for K in the third column of Table II we have the true values for the velocity in the last column. These were the data used in calculating the chances of collision.

TABLE II.

Diameters and Velocities of the Three Groups of Leucocytes.

Cell group.	Average diameter.	Relative velocity × per cent of total cells.	True velocity.
	μ		*cm. per hr.*
1	7.6 ± 0.04	$58.0\,K \times 0.32 = 18.6\,K$	1.20 ± 0.035
2	8.9 ± 0.016	$79.5\,K \times 0.39 = 31.0\,K$	1.64 ± 0.048
3	10.8 ± 0.034	$108.5\,K \times 0.29 = 31.4\,K$	2.24 ± 0.066
Average velocity.....		$81.0\,K$	1.67 ± 0.05

From these data the density of the cells may be determined by equation (3).

$$D - d = \frac{5.1 \times 1.64}{8.9^2} = 0.105$$

$$D = 1.007 + 0.105 = 1.11$$

Comparison of the Rates of Phagocytosis of Quartz Particles of Three Different Sizes.

Experiments 1 and 2.—The three quartz suspensions were washed once with distilled water and were then allowed to settle at room temperature. After the sharp boundary of the suspension had settled 3 or 4 cm. from the top of the liquid, a definite amount, about 2 cm., was siphoned off the top of each suspension as already described. From the minimum and maximum velocities so obtained, the average velocity is calculated by equation (2) (Table III). The diameter is then given by equation (3), and the chances of collision with the leucocytes by the formula

$$R_{\text{total}} = \frac{R_a P_a + R_b P_b + R_c P_c}{100} \qquad (5)$$

where R_a, R_b, and R_c are the chances of collision with the three groups of cells a, b, and c; and P_a, P_b, and P_c, the percentages of cells in these groups. The velocity of the 2.55μ particles being most nearly equal to that of the cells, these particles have the smallest chance of collision, as shown in Table III. Under the microscope these suspensions appeared as absolutely uniform as any suspension of irregular particles could be (Fig. 4).

The phagocytic mixtures were prepared as follows: 0.2 cc. of washed leucocyte suspension in 0.9 per cent sodium chloride, plus 0.15 cc. of quartz suspension in 0.9 per cent acacia plus 0.1 cc. of fresh serum plus 0.05 cc. of M/10 phosphate mixture ($[H]^+ = 3 \times 10^{-8}$) plus 0.05 cc. of 4.5 per cent sodium chloride. The acacia was added to prevent agglutination of the quartz. The phosphate helped to maintain the hydrogen ion concentration equal to that of blood serum. Frequent colorimetric measurements of $[H]^+$ at the close of the experiments showed no appreciable change in reaction from the original. Without serum there is very little phagocytosis.

TABLE III.

Comparison of Theoretical and Experimental Rates of Phagocytosis of Three Sizes of Quartz Particles.

Average diameter of particles.			2.92 μ	2.55 μ	1.85 μ	Ratio.
Velocity per hr. in cm.	Minimum		2.51	1.89	1.00	
	Maximum		3.54	2.73	1.44	
	Average		2.81	2.14	1.13	
Chances of collision			151.8	61.8	76.5	1:0.41:0.51 (Theoretical.)
Experiment 1 (Fig. 5)	K		0.078	0.033	0.05	1:0.42:0.64 (Experimental.)
	Average per cent of error	Assumed..	9.0	4.0	7.0	
		Expected.	7.0	5.0	5.7	
Experiment 2 (Fig. 6)	K		0.38	0.33	0.22	1:0.87:0.58 (Experimental.)
	Average per cent of error	Assumed..	7.0	3.1	4.4	
		Expected.	8.0	7.6	6.7	
						1:0.64:0.61 (Average experimental.)

The above mixture was incubated at 37°C. in small glass-stoppered vials 6 mm. in outside diameter, which were rotated about their horizontal axis once in 2 minutes. The speed of rotation was kept constant in any one experiment. Since the tubes were so small and were held horizontal, there was very little stirring by the air bubble included at one end of the tube. Counts of the number of particles outside the phagocytes per 0.02 mm.^{3} of solution were made at intervals. The logarithms of these counts were then plotted as ordinates against time as abscissæ. If phagocytosis follows the laws of monomolecular reactions these points should lie on a straight line of slope K, where K is the velocity of the reaction. A summary of Experiments 1 and 2 is given in Table III and the experimental points are plotted in Figs. 5 and 6.

In Experiment 1, K and logarithm A were determined by the method of least squares; in Experiment 2, they were measured graphically. In the latter instance K was nearly ten times as high as in the former. This may have been due to a difference in the cells or in the serum. The quartz suspensions used were identical.

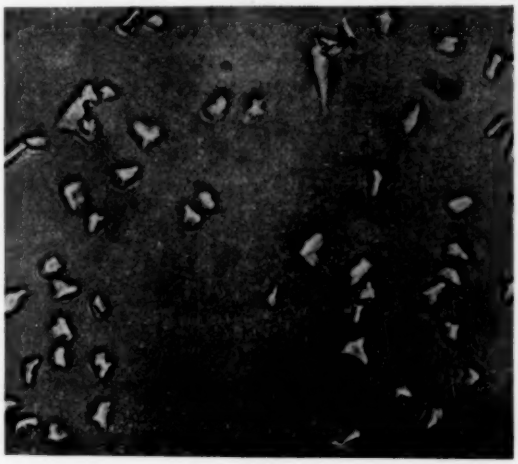

FIG. 4. Photograph of quartz suspension 4.6 μ in diameter, to show uniformity in size of particles.

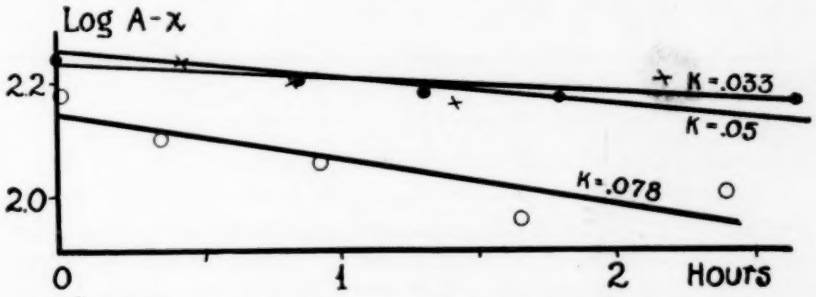

FIG. 5. Experiment 1. Ordinates represent logarithms of the number of particles not taken up by leucocytes plotted against time as abscissæ. K is equal to the slope of the graph. Quartz particles of three different sizes are compared, designated as in Fig. 6. Experimental ratios, 1:0.42:0.64; theoretical ratios, 1:0.41:0.51. K determined in this experiment by method of least squares. See Table III.

In Table III is also given the average per cent of error which was assumed in the experimental points in order to fit them to the equation and the average probable error involved in counting the particles for the experimental points. The latter value was calculated by the expression $\frac{1.05}{\sqrt{N}}$, where N was the number of particles counted.[5] The assumption that the points lie on a straight line is justified by

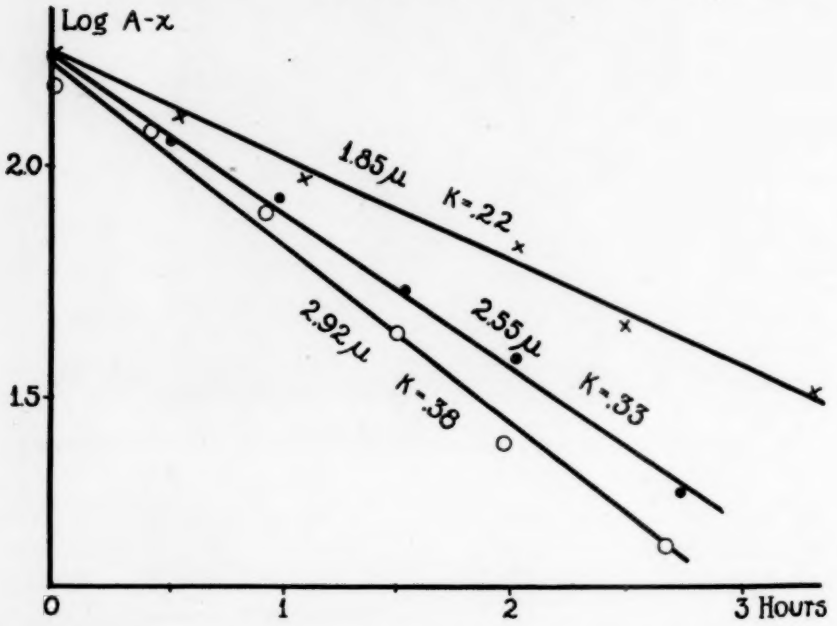

FIG. 6. Experiment 2. Legend as in Fig. 5. Experimental ratios, 1: 0.87: 0.58; theoretical ratios, 1: 0.41: 0.51. K's determined graphically. See Table III.

[5] The error of a set of observations varies inversely as the square root of the number of observations (Tuttle, L., The theory of measurements, Philadelphia, 1916, 219). The constant 1.05 ± 0.049 was determined for counting particles from calculations of the dispersion of extensive counts made on fourteen different suspensions. The dispersion of the fourteen determinations was ±0.19. The constant was also determined from counts made on forty-eight different leucocyte suspensions and gave 1.07 ± 0.05, the dispersion of the individual determinations being ±0.34.

the fact that the error assumed is in general no larger than the probable error. Reference to Table III will show that the ratios between the three experimental constants agree fairly well with the theoretical ratios.[6]

Experiments 3 and 4.—Two more experiments were tried in which only the largest and smallest particles were compared. New suspensions were prepared from the same stock and in the same way as before. The sizes are accordingly somewhat different. The rates of phagocytosis are compared with the chances of collision in Table IV, and the experimental points are plotted in Figs. 7 and 8.

TABLE IV.

Comparison of Theoretical and Experimental Rates of Phagocytosis of Two Sizes of Quartz Particles.

Average diameter of particles.		3.18 μ	1.73 μ	Ratios.
Velocity per hr. in cm.	Maximum	3.95	1.91	
	Minimum	3.07	1.21	
	Average	3.33	1.41	
Chances of collision		286	51	1:0.18 (Theoretical.)
Experiment 3 (Fig. 7)	Initial K	0.133	0.046	1:0.35 (Experimental.)
	Later K	0.84	0.174	1:0.21 (")
Experiment 4 (Fig. 8)	Initial K	0.265	0.1	1:0.38 (")

In Experiment 3 the reaction starts very slowly and for some unknown reason the cells become changed or the medium becomes more favorable so that they can ingest particles more readily. Each reaction is, therefore, made up apparently of two distinct parts. If the change in the cells in the two tubes is comparable the ratio of the K's should be the same for the later K's as for the initial K's. This turns out to be true and both ratios agree well with the theoretical ratio. The inclusion of the later K's is admittedly somewhat arbitrary and is given merely for what it is worth.

The graphs in Fig. 7 for the 3.18 μ particles show considerable irregularities but the agreement of the initial K's is very good and the ratio of the initial K's for the two sizes of particles agrees fairly well with the theoretical.

[6] A discussion of the significance of a constant K in view of the gradual agglutination of cells is postponed to the next paper of this series.

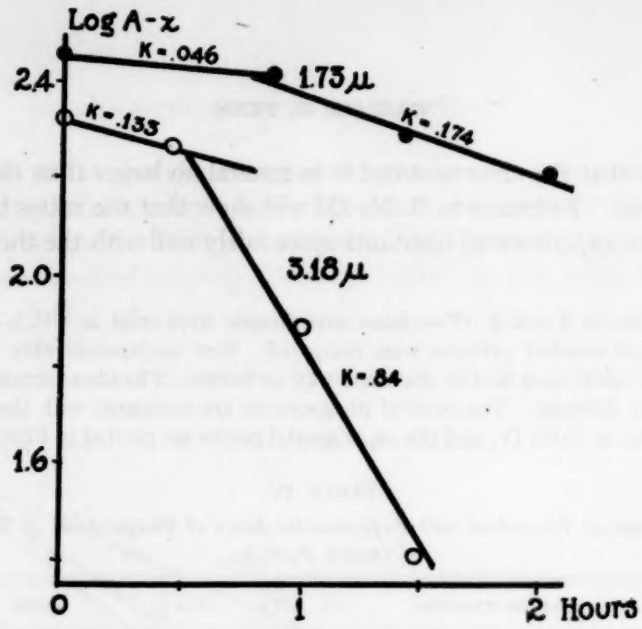

FIG. 7. Experiment 3. Ordinates represent logarithms of numbers of particles not ingested. Since K is not constant the initial K's only can be used in comparisons. Experimental ratio, 1: 0.35; theoretical ratio, 1: 0.18. Ratio of K from the end of the experiment is 1: 0.21. The two sizes of quartz particles correspond to the largest and smallest in Figs. 5 and 6. See Table IV.

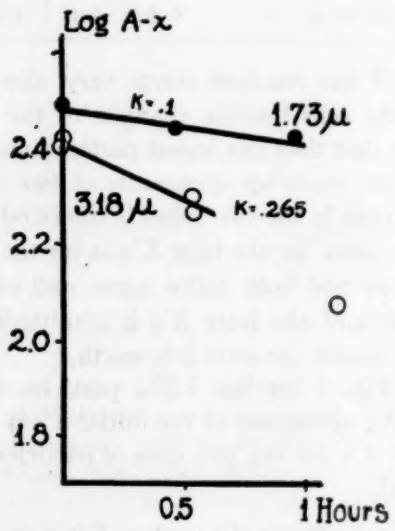

FIG. 8. Experiment 4. Repetition of Experiment 3. Only the initial K's give any basis for comparison. Experimental ratio, 1: 0.38; theoretical ratio, 1: 0.18. See Table IV.

Experiments 5 and 6.—In two more experiments on quartz particles the suspensions used were less uniform because the large particles were removed by centrifugalization. The diameters of these particles were obtained by direct microscopic measurement. Frequency curves so obtained are plotted in Fig. 9. Each frequency curve was then divided into a number of groups and the chances of collision calculated between each one of the quartz groups and the three groups of cells by equation (5). The chances of collision for any one suspension is, therefore, equal to the sum of the products of the chances of collision of each quartz group by the per cent of particles in that group, or

$$R_{\text{total}} = \frac{R_{a_1}P_aP_1 + R_{a_2}P_aP_2 + R_{a_3}P_aP_3 + \ldots + R_{c_3}P_cP_3}{100}$$

where P is the per cent of cells or particles in the designated group, R the chance of collision; the subscripts 1, 2, and 3 refer to groups of particles while a, b, and c refer to groups of cells.

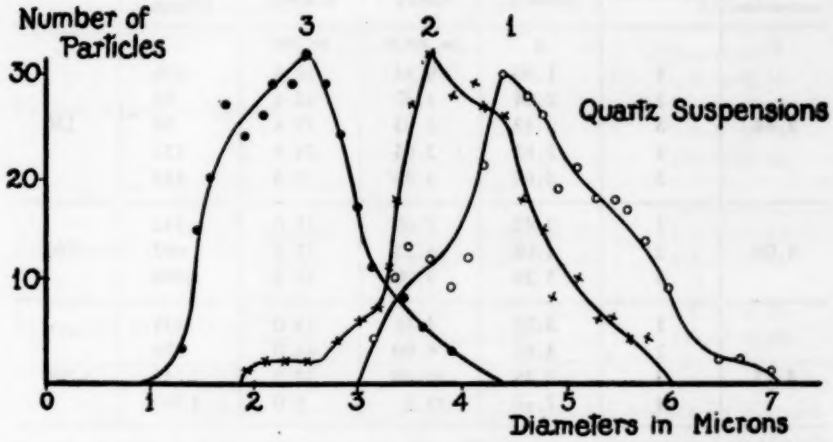

FIG. 9. Frequency curves where ordinates represent number of particles and abscissæ represent diameters of particles in microns. Data from microscopic measurements of three quartz suspensions used in Experiments 5 and 6. Average diameters equal 2.44 μ, 4.08 μ, and 4.63 μ. Points plotted are experimental points smoothed by averaging each ordinate with the two adjacent ordinates. Plotted to such a scale that the areas subtended by each graph are equal. See Table V.

The calculation in this case is much more laborious and the results are not so satisfactory. Moreover, in these experiments, which were the first comparisons attempted, larger test-tubes (8 mm. inside

diameter) were used for incubation, and there was more irregular stirring by the air bubble which moved up and down the rotating tube. Under these circumstances the explanation offered for the different rates of ingestion of the three suspensions seems satisfactory.

The data used in calculating *R* are given in Table V. The velocities obtained for each group were the weighted averages of all the separate velocities in the group, not the velocity corresponding to the average diameter. The group diameters of the two larger suspensions are the

TABLE V.

Chances of Collision of Leucocytes with Three Sizes of Quartz Particles as Prepared in Experiments 5 and 6.

Average diameter of suspension.	No. of group.	Group diameter.	Group velocity.	Particles in group.	Group chances of collision.	Total *R*.
μ		μ	*cm. per hr.*	*per cent*		
	1	1.60	0.84	18.8	106	
	2	2.04	1.37	23.4	59	
2.44	3	2.42	1.93	19.4	54	130
	4	2.83	2.64	24.8	125	
	5	3.68	4.46	13.3	413	
	1	2.82	2.69	11.6	132	
4.08	2	4.05	5.23	71.5	602	697
	3	5.28	9.00	16.8	1,500	
	1	3.70	4.38	19.0	431	
	2	4.67	6.90	44.0	979	
4.63	3	5.45	10.00	32.0	1,745	1,299
	4	7.40	18.5	5.0	4,560	

average diameters of the group. For the smaller suspension they are calculated from the average velocity. The difference in the result is too small to have any appreciable effect. The latter method is more accurate because it is the square of the diameters which enters into the equation. The error in calculating must be larger for the smallest suspension because in this case the group velocities nearly coincide with the velocities of the groups of cells. The true chance of collision is, therefore, somewhat higher than the calculated figure. This could be avoided by the use of calculus, treating the frequency

curves in Fig. 8 as normal frequency curves, but the resulting expression would be too difficult to work with.[7]

Since the group chances of collision are not all equal in the same suspension the chances of collision as a whole will tend to decrease as those particles with the greatest R are taken up first. It can be shown that R decreases most rapidly for the 4.63 μ particles. The ratios, therefore, increase. This ratio has been calculated for the times in the experiment when the 4.63 μ particles are 50, 75, and 90 per cent ingested (Table VI).

TABLE VI.

Comparison of Theoretical and Experimental Rates of Phagocytosis of Three Sizes of Quartz Particles.

Average diameter of particles.		4.63 μ	4.08 μ	2.44 μ	Ratios.
					(Theoretical.)
Per cent of particles ingested.....	0				1:0.54:0.10
	50				1:0.62:0.13
	75				1:0.7 :0.16
	90				1:0.85:0.24
					(Experimental.)
Experiment 5 (Fig. 10)	Initial K.....	0.76	0.70	0.33	1:0.92:0.43
	Total K.....	0.580	0.477	0.196	1:0.82:0.34
Experiment 6 (Fig. 11)	Initial K.....	0.44	0.27	0.06	1:0.61:0.14
	Total K.....	0.57	0.348	0.134	1:0.61:0.24

In Figs. 10 and 11 are plotted the experimental points in Experiments 5 and 6 respectively, and the results are summarized in Table VI. For comparison, the initial and the total K's have been calculated. The latter are calculated by the method of least squares and the former directly from the experimental figures. The agreement between these experimental ratios and the expected ratios seems remarkably good when the many complicating factors involved in this type of experimentation are taken into consideration.

[7] The writer wishes to express his gratitude to Professor R. G. Wilson, Massachusetts Institute of Technology, for information on this point and also for valuable criticism of this work.

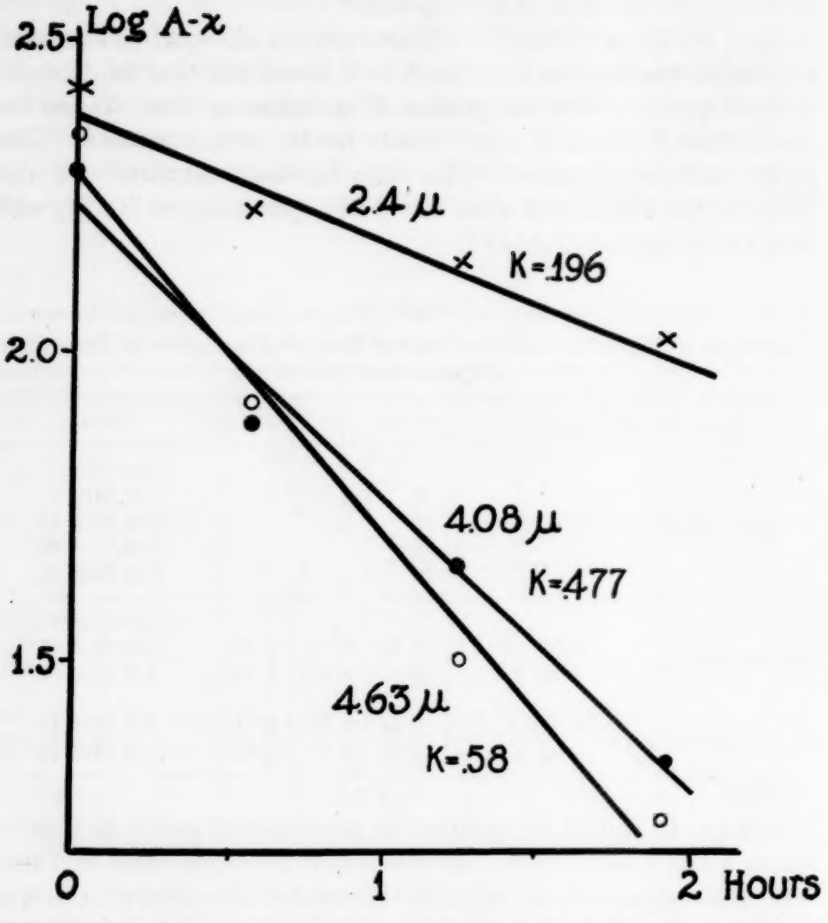

FIG. 10. Experiment 5. Logarithms of number of particles not ingested plotted as ordinates against time as abscissæ. See Table VI for comparison of theoretical and experimental ratios and values of initial K's. Total K's, as plotted, were determined by method of least squares.

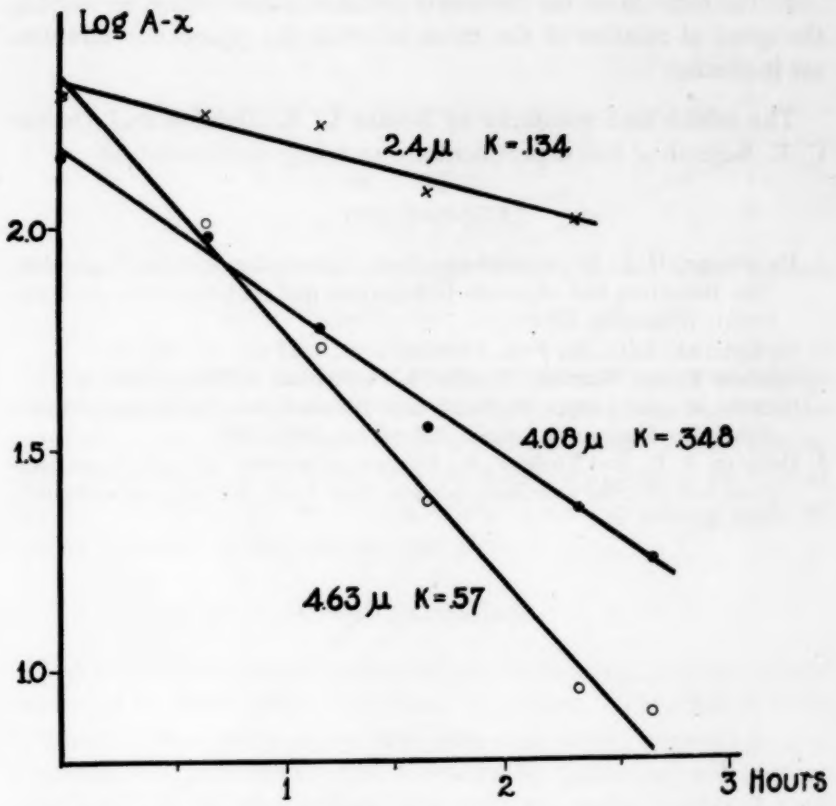

FIG. 11. Experiment 6. Repetition of Experiment 5, Fig. 10. See Table VI for comparisons of theoretical and experimental ratios and values of initial K's.

SUMMARY.

1. A new quantitative method of measuring phagocytosis of solid particles is described.

2. A method of calculating the chances of collision between leucocytes and quartz particles of different sizes is developed.

3. The speed with which three suspensions of different sized quartz particles should be ingested by leucocytes is predicted from the calculated chances of collision, and the prediction is verified experimentally.

4. The formula for the chances of collision is also verified by varying the speed of rotation of the tubes in which the phagocytic mixtures are incubated.

The advice and assistance of Doctor C. K. Drinker and Doctor C. K. Reiman of this department are gratefully acknowledged.

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THE PHAGOCYTOSIS OF SOLID PARTICLES.

II. Carbon.

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In a former paper (1) some determinations were reported of the rates of ingestion of three different sizes of quartz particles by leucocytes of the rat. It was shown that the rate of phagocytosis depends upon the chances of collision between a leucocyte and a particle of quartz. In this paper similar experiments on the phagocytosis of carbon particles of two sizes are reported.

Carbon Suspensions.

The method of preparing the carbon suspensions was essentially the same as that already described for quartz. The carbon used was one of the charcoals prepared from coal during the war by the Chemical Warfare Service and activated by treatment with superheated steam. It was washed repeatedly in distilled water during the process of removing, by means of the centrifuge, the particles too small to use, and was washed once each time before using. It may, therefore, be considered free from easily soluble material.

The carbon suspensions were considerably more sensitive to agglutination than the quartz. It was, for example, impossible to resuspend a suspension of carbon, after it had settled out either by gravity or centrifugal force, without getting some agglutination. The suspensions were agglutinated at once by the addition of tap water and they were somewhat more stable in slightly alkaline than in slightly acid solutions. Non-sterile suspensions agglutinate more readily than sterile ones. Acacia must be added to a suspension to keep it uniform and stable for more than a few hours. Fortunately

the hemoglobin and serum proteins act fairly efficiently as protective colloids, and the normal reaction of blood, at which these experiments were done, is slightly alkaline. Otherwise, it would be impossible to keep the particles distinct in 0.9 per cent sodium chloride with the leucocytes. Even so, the agglutination of the carbon was a very serious complicating factor in these experiments, more so than with quartz.

In view of the readiness with which these uniform suspensions agglutinate, it is a significant fact that in the stock suspensions, from which the large particles have not yet been removed, all the particles are never agglutinated even after being kept for nearly a year without acacia. It would seem as if coagulation must reach an equilibrium and stop. It is certain that there is always an abundance of the smallest sized particles which are not clumped and can readily be separated from the larger particles and the clumps by settling.

Chances of Collision with Leucocytes.

The diameters of the particles in the two suspensions and the diameters of the leucocytes were measured microscopically. The variation in these diameters is shown in three frequency curves in Fig. 1. On account of the lack of uniformity of these suspensions the chances of collision between leucocytes and particles could not be calculated from the average diameter and velocity of all the particles in the suspension. It was, therefore, necessary to divide each frequency curve, as given in Fig. 1, into sections and calculate the chances of collision, R, for each possible combination, by the formula $R = (C + P)^2 (Vp - Vc)$ (1). Here the first term is the square of the sum of the diameters of the cell and particle, and allows merely for the larger target offered by a large particle. The second term is the difference in velocity between the cells and particles. Since the velocities of the cells and particles in this case were nearly equal, a small error in Vp or Vc would make a large error in R. It was, therefore, necessary to divide the carbon suspensions into six groups each to obtain sufficient accuracy in the calculation. The leucocytes were divided into three groups as described in the previous paper. The total chances of collision were then given by the sum of the 18 (6×3)

group chances of collision, each multiplied by the percentage of cells and of particles in that particular group. The data from which the chances of collision were taken are given in Table I together with the chances of collision of each suspension.

It will be noted that in calculating the chances of collision the velocity was determined experimentally and was not calculated from the diameter and density. This was done because of the fact that the velocity of carbon particles is not proportional to the density of dry carbon which is full of capillary spaces, but to its density with

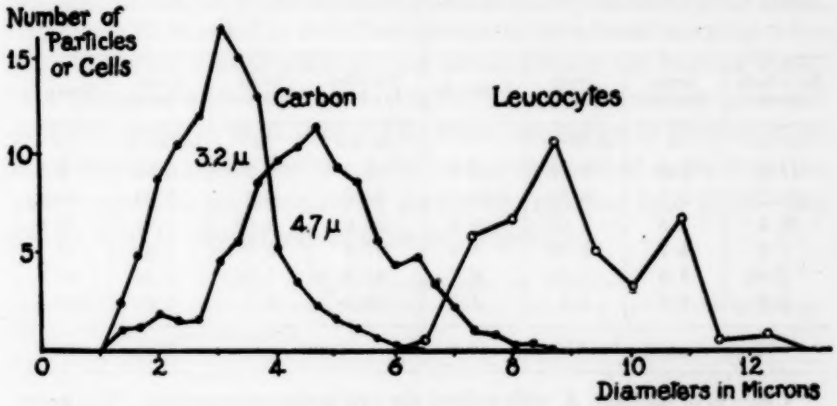

FIG. 1. Frequency curves showing the variation in the diameters of two carbon suspensions and the leucocytes. Curves are plotted on such a scale that the subtended areas are approximately equal. Ordinates represent number of particles for a given interval of the abscissæ, which represent the diameters in microns. Points plotted are experimental points. In the case of the carbon suspensions they are smoothed by averaging each point with the two adjacent points.

these spaces full of water; and the latter is not easily determined. However, having obtained the velocity of each group directly, without using any value for the density, it is possible to reverse the process and calculate both the density and the volume of the capillary spaces, as a check on the accuracy of the measurements.

By a simplification of Stokes's law for the velocity, V, of a particle of diameter, P, and density, D, in a medium of density 1.00 and viscosity 0.010 under the acceleration of gravity, we obtain the equation

$$D = 1 + \frac{5.1\ V}{P^2}$$

Substituting in this equation values for the average velocities and average diameters of the two suspensions (0.87 ± 0.12 and 2.19 ± 0.18 cm. per hour for the 3.2μ and 4.7μ respectively), and solving for D, we obtain 1.43 ± 0.06 and 1.50 ± 0.04. This is the density with capillary spaces full of water.

TABLE I.*

Chances of Collision between Leucocytes and Carbon Suspensions.

3.2 μ				4.7 μ			
Particles in group.	Group diameter.	Group velocity.	Group *R*.	Particles in group.	Group diameter.	Group velocity.	Group *R*.
per cent	μ	*cm. per hr.*		*per cent*	μ	*cm. per hr.*	
14.3	1.8	0.27	24.9	5.3	1.9	0.35	9.1
36.2	2.7	0.59	58.6	14.0	3.2	0.99	16.2
39.4	3.5	1.01	46.6	29.6	4.08	1.61	17.9
7.3	4.7	1.78	5.1	27.9	5.0	2.39	35.6
2.04	5.6	2.58	3.7	13.3	5.94	3.36	48.6
1.0	6.8	3.8	5.2	10.0	6.8	6.5	120.8
Total chances of collision = 144.1				Total chances of collision = 248.2			

* Chances of collision, R, with cells of the two carbon suspensions. The group diameters were obtained by averaging the squares of the individual diameters as measured in the microscope. Group velocities were calculated in proportion to the squares of the group diameters and so that their weighted average velocity should be equal to the average velocity of the suspension as determined experimentally by the stop-cock method; *i.e.*, to 0.87 ± 0.12 and 2.19 ± 0.18 cm. per hour for the 3.2μ and 4.7μ suspensions, respectively. The R group or chance of collision is given by the formula

$$R \text{ group} = (R_{1a}\ P_a + R_{1b}\ P_b + R_{1c}\ P_c)\ \frac{P_1}{100}$$

where P equals the per cent of particles or cells in designated group, subscripts a, b, and c refer to the three groups of cells, and the numerical subscripts refer to the groups of particles.

From these figures may be calculated the cubic centimeters of capillary space, x, in the carbon per gram of dry weight, as follows:

$$D = \frac{1.81 + 1.81x}{1 + 1.81x}$$

1.81 being the density of dry carbon. Whence, introducing the calculated values for D and solving, we get $x = 0.49 \pm 0.09$ and 0.34 ± 0.07, the average being 0.42 cc. of capillary space per gram of carbon. Though the variation is relatively large it is indicative of the general accuracy of the calculations that this is the exact figure given for the volume of the capillary spaces in charcoal by Cude and Hulett (2), from a study of a large series of charcoals used by the Chemical Warfare Service.

It is possible, then, to predict the behavior of the leucocytes toward the particles of these suspensions when they are incubated together. The predictions are: (1) that the large particles will be taken up more rapidly than the small particles, the initial ratio of the rates of ingestion being as 248 : 144 = 1 : 0.58; and (2) that this ratio will probably increase with time. The latter prediction is illustrated by the following figures for the ratio of the chances of collison of the two suspensions at such times during the reaction that 25, 50, and 75 per cent of the larger particles are ingested.

Change in Ratio Predicted during Reaction.

4.7 μ particles ingested.	Ratio. $R_{4.7\,\mu} : R_{3.2\,\mu}$
per cent	
0	1:0.58
25	1:1.07
50	1:1.26
75	1:1.42

This change in ratio is largely due to lack of uniformity of the 4.7μ suspension, the largest particles having an abnormally high chance of collision as shown in Table I. The prediction is, however, merely a probability since the assumption upon which it is based, namely that the leucocytes remain unchanged in concentration, can never be completely true. However, it may be noted that the experiments largely verify these predictions.

Method of Measuring Phagocytosis.

The detailed technique of these experiments is given in a previous paper (1). It is sufficient to state here that mixtures consisting of washed leucocytes from rats, carbon suspension, and serum were

incubated at 37°C. in small test-tubes (8 mm. inside diameter), placed horizontally on a drum revolving uniformly about its horizontal axis. The speed of rotation was usually about 15 revolutions per minute. In these experiments no precautions were taken to prevent an air bubble from traveling up and down in the tube as it revolved. Samples were removed at intervals and counts were made of the *number of particles not ingested* by the leucocytes.

The measure of phagocytosis desired is, then, the per cent of the collisions occurring between cells and leucocytes which result in ingestion. But the number of leucocytes being the same in any one experiment, the *relative* number of collisions is proportional only to the number of particles. If the percentage of collisions resulting in ingestion is constant and the concentration of cells does not change, the rate of ingestion will always be proportional to the number of particles still free to collide, and the velocity constant, K, will be given by the equation for a monomolecular reaction, $K = \frac{1}{t} \log \frac{A}{A - x}$ where A is the original number of particles and x is the number ingested in time, t. Here the relative value of K is dependent only on the percentage of collisions resulting in ingestion, and is, therefore, the measure of phagocytosis desired. K has been determined in practice by plotting the logarithms of the number of particles still free, *i.e.* $\log A - x$, against time and determining the slope graphically. The points should lie on a straight line if K is constant. This is more accurate than calculating K directly from the formula, since the formula attaches special importance to A which is known no more accurately than the rest of the points.

Where K is not constant the initial value of K only is determined. A *decrease* in K with time may be due to:

1. Decrease in the number of cells by agglutination.[1]
2. Decrease in the capacity of cells for particles.
3. Lack of uniformity in the suspensions resulting in decrease in chances of collision as larger particles in each suspension are ingested more rapidly than the small particles.

[1] It can be shown that in calculating the chances of collision the decrease in the number of cells by agglutination is not compensated for by the increase in velocity and size of the resulting clumps.

4. Decrease in activity of cells due to internal changes or changes in environment.

An *increase* with time may be due to:

5. Increase in velocity of cells caused by increased density after ingestion of particles.

6. Increase in activity of cells by internal changes or changes in environment.

Of these factors, No. 3 can be predicted by measurements of the particles in the suspension and has no effect if thoroughly uniform suspensions are used. Nos. 4 and 6 are probably negligible. Nos. 1, 2, and 5, then, are the significant factors. No. 1 is presumably the same for each carbon suspension in any one experiment so that it does not vitiate the comparison. Nos. 2 and 5 are small if the number of cells is large. In the majority of experiments there were from one to two particles per cell in the most concentrated suspension, probably never over three, and frequently less than one. Since each cell could take up perhaps five large particles and fifteen small ones they could hardly be appreciably filled up. It seems valid to use the total K, where K is constant, as well as the initial K for comparison. Usually the total K does not differ from the initial K by more than the experimental error. The latter is, of course, the more inaccurate as it depends upon only two points, while the total K is the average of all points. It is, perhaps, surprising that K should be so constant. This must indicate that the disturbing factors are in such cases comparatively small and that those causing an increase in K partly compensate for those causing a decrease.

In Table II the results of five such comparisons of rates of ingestion of the two suspensions of carbon are given. The results for three of these experiments are plotted in Figs. 2, 3, and 4. The other two experiments are somewhat less reliable and are included merely for completeness.

It must be admitted that the agreement between the theoretical initial ratio and the average experimental ratio is better than the variation in the individual ratios warrants. It will be seen that in Figs. 2 and 3 the second prediction is fulfilled as well as the first, for the slope of the curve representing the ingestion of the 4.7μ particles continually decreases, while that of the 3.2μ particles remains con-

TABLE II.

Comparison of Experimental and Theoretical Rates of Phagocytosis.

Experiment No.	Initial K.		$\frac{K_{3.2\,\mu}}{K_{4.7\,\mu}}$	
	4.7 μ	3.2 μ		
1	0.36	0.12	0.30	Fig. 2
2	0.60	0.38	0.63	" 3
3	0.21	0.16	0.76	" 4
4	0.125	0.10	0.80	Not figured.
5	0.88	0.32	0.36	" "
Average			0.57	
Theoretical			0.58	

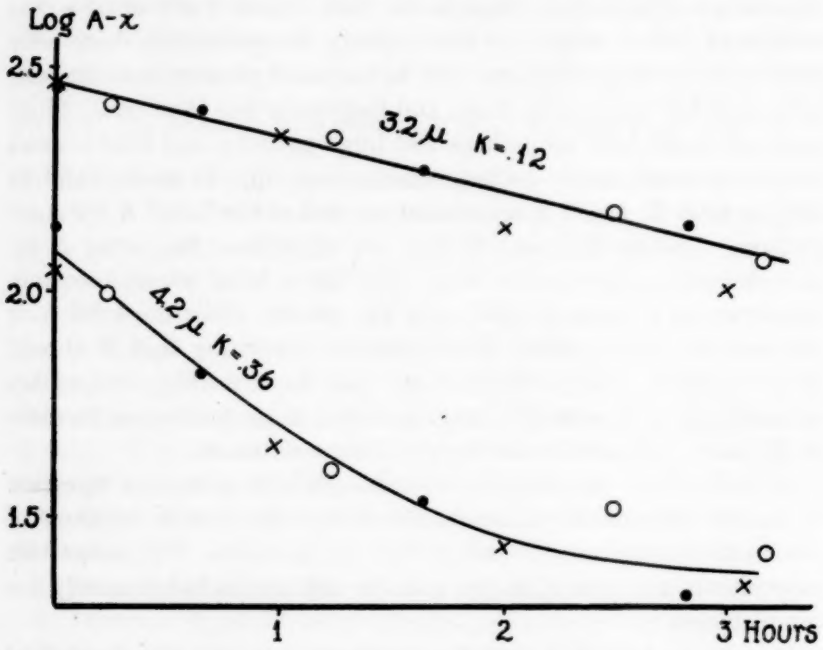

FIG. 2. Ordinates represent logarithms of the number of particles not yet ingested. Abscissæ represent time in hours since the beginning of the experiment. Points from three separate but simultaneous experiments are plotted for each curve. Experimental ratio $\frac{K_{3.2\mu}}{K_{4.7\mu}}$ is 0.3; theoretical ratio is 0.58. The fact that $K_{4.7\mu}$ is not constant causes this ratio to increase as predicted.

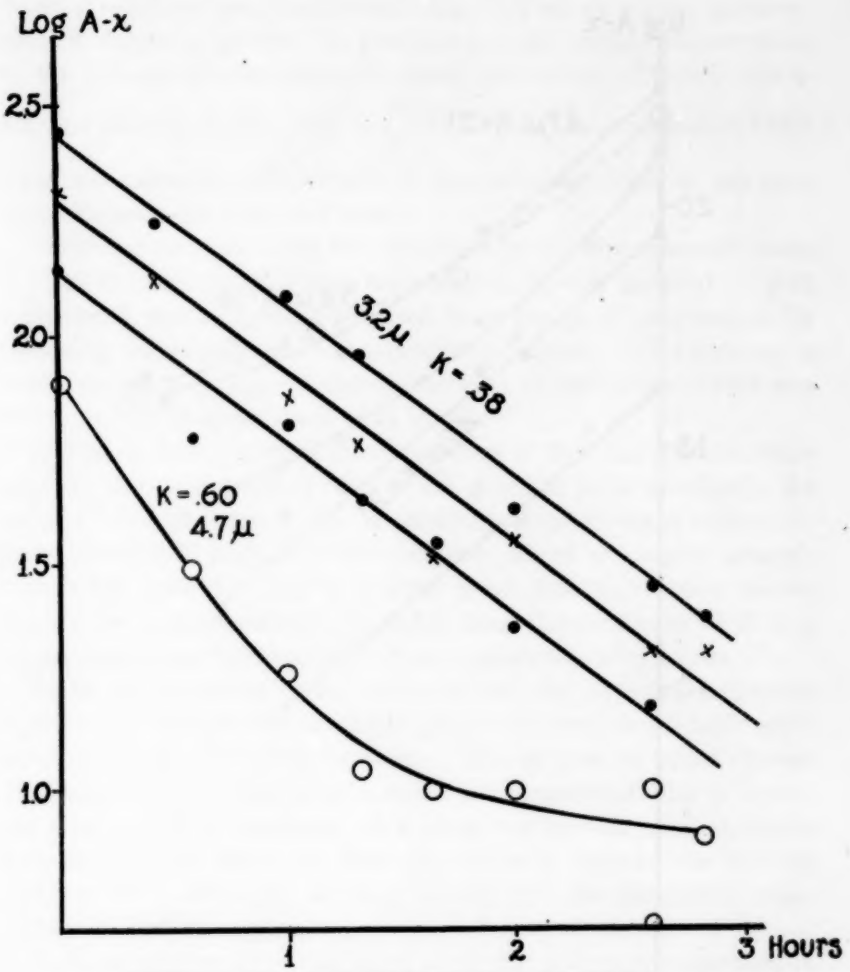

FIG. 3. Curves showing the rates of ingestion of 3.2μ and 4.7μ carbon particles. The logarithms of the number of particles not ingested are plotted (ordinates) against time (abscissæ). All three concentrations (4, 3, and 2) of the 3.2μ particles give $K = 0.38$ approximately. Experimental initial ratio $\frac{K_{3.2\mu}}{K_{4.7\mu}} = 0.63$; theoretical ratio is 0.58. This ratio increases with time due to the decrease in $K_{4.7\mu}$ as predicted.

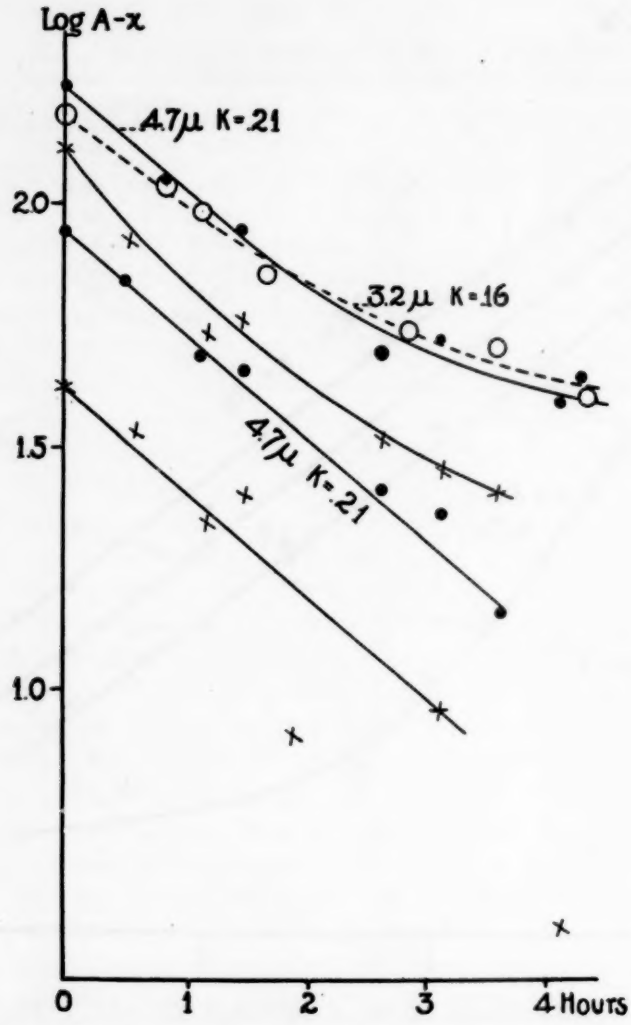

FIG. 4. Curves showing logarithms of numbers of 3.2μ and 4.7μ particles not ingested (ordinates) plotted against time (abscissæ). Four concentrations (4, 3, 2, and 1) of the 4.7μ particles were used. The best initial K is 0.21 which is well satisfied except in one case. Experimental ratio $\frac{K_{3.2\mu}}{K_{4.7\mu}} = 0.76$; theoretical ratio is 0.58. This ratio does not increase during the experiment.

stant throughout the experiment. Fig. 3 is an exception, however, since K decreases for the 3.2μ particles and the higher concentrations of the 4.7μ particles in about the same proportion. There is, therefore, no increase in the value of $\frac{K_{3.2\mu}}{K_{4.7\mu}}$. In the two experiments which were not calculated, Experiment 4 showed an increase in this ratio while Experiment 5 showed none.

There are two causes for the variations in the experimental result: (1) The suspensions used were not identical, for the material for each experiment was separately prepared from the stock suspensions by removing the large particles by centrifugalization. (2) There was a slight tendency to aggregation on the part of the carbon which was more marked in some cases than in others.

In Fig. 3, three different concentrations of the 3.2μ particles were used, in the proportions 4 : 3 : 2, all of which have practically the same K throughout. In Fig. 4, similarly, four different concentrations of the 4.7μ particles were compared, all of which give approximately the same K. This is a better proof that the reaction follows the law for a monomolecular reaction than the constancy of K in a single experiment because of gradual aggregation of the cells.

While the agreement between theoretical and experimental ratios in these experiments is admittedly rough, it seems surprisingly good considering the difficulties involved. It is at least accurate enough to discourage any attempts to measure the greater difficulty of ingesting a large particle compared to a small one by this method, which was the original object of these experiments, because the limiting factor is the availability of the particles, not the phagocytic capabilities of the leucocytes.

It should perhaps be emphasized here that a certain proportion of the particles counted as phagocytized are merely stuck on the outside of clumps of cells where it is impossible to distinguish them from those inside. This does not occur with dead cells, however, and is rightly regarded as the first stage in phagocytosis.

Phagocytosis of Bacteria.

It is interesting to inquire at this point whether the phagocytosis of bacteria also follows the law for a monomolecular reaction, as does

the phagocytosis of solid particles. This can only be determined if the number of bacteria ingested in proportion to the number of bacteria present is known. In most experiments on phagocytosis of bacteria no counts have been made of the total number of bacteria present. The only data known to the writer from which this point can be determined are those of Madsen and Watabiki (3) and of Ledingham (4). The former measured the number of bacteria ingested as a function of time. Their curves follow the law of a monomolecular reaction fairly well if all the bacteria not ingested at the close of the experiment, *i.e.* when phagocytosis has ceased, are disregarded. The rate of phagocytosis is, therefore, not proportional to the concentration of the bacteria present.

Ledingham did for phagocytosis of bacteria what was done in Figs. 3 and 4 for the phagocytosis of carbon. He measured the number of bacteria ingested in unit time by a given number of cells from a series of bacterial suspensions of varying known concentrations. If the reaction had been of the monomolecular type the percentages ingested at each concentration should have been equal, just as all the K's in Figs. 3 and 4 were equal for the same size particle. He found, however, that as the concentration of bacteria decreased, the percentage ingested first increased and then decreased. There is, therefore, no evidence that the phagocytosis of bacteria follows the law for a monomolecular reaction. If, as Ledingham believes, the comparatively slight decrease in the percentage ingested in low concentrations of bacteria is due to experimental error, the decrease in higher concentrations may be due to increasing concentrations of some substance given off by the bacteria which is toxic to the cells.[2]

[2] In these experiments Ledingham's object was to prove that phagocytosis of bacteria obeys Freundlich's exponential formula for adsorption, but it is hard to see how the formula could have any significance for phagocytosis even if applicable. He also tries to show that the adsorption of opsonin from serum by bacteria follows the same law, but his measure of the concentration of opsonin adsorbed is mathematically incorrect. He assumes that the opsonin adsorbed by the bacteria is proportional to the difference in the number of bacteria ingested by a given number of leucocytes in the presence of an opsonin solution, before and after that solution has been treated with a given bacterial emulsion (which adsorbs some of the opsonin) and freed from it by centrifugalization. His own figures show, however, as he himself observes, that the number of bacteria ingested is

Ledingham himself describes "lytic changes" in these leucocytes which he ascribes to the "leucotoxic action of unneutralised bacterial extract." This supposition might also explain the fact observed by Madsen and Watabiki that the rate of phagocytosis decreases more rapidly than the concentration of bacteria, the cumulative effect of the toxic substance finally preventing phagocytosis altogether before all the bacteria are ingested.

Other Experiments on Phagocytosis of Carbon.

The Effect of Serum.

Hamburger (5) found that the carbon particles which he used with horse leucocytes were ingested just as well in pure sodium chloride as in sodium chloride and serum. Fig. 5 shows that this was distinctly not the case in these experiments. In this figure Hamburger's measure of phagocytosis was used; *i.e.* the percentage of cells containing carbon. These values are plotted as ordinates in Fig. 5 against time as abscissæ. Two controls, one without serum and one with serum, heated to 56°C. for 40 minutes, showed almost no phagocytosis. This is the usual result obtained by previous workers on phagocytosis of solid particles, although there is considerable disagreement. Hamburger finds serum necessary for the phagocytosis of starch but not of carbon.

Porges (6) found that starch is taken up from isotonic solutions without serum as well as with serum, but the accelerating effect of serum became evident in hypertonic solutions.

Ouweleen (7) concludes that two substances are necessary for the phagocytosis of starch: (1) a thermolabile constituent of serum which is absorbed on the starch and causes adhesion of the starch to the leucocyte; and (2) a substance causing the adhering particle to be

not a linear function of the opsonin concentration. From his figures for the number of bacteria ingested in the presence of known concentrations of opsonin, it is possible to calculate graphically what the true concentration of opsonin must be after adsorption to account for the observed number of bacteria ingested. Using figures so obtained the adsorption formula no longer applies. In only one experiment, however, were his data sufficiently complete to make this method of analysis possible.

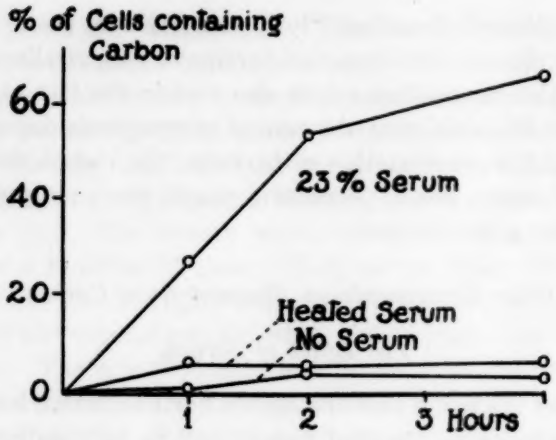

FIG. 5. Curves showing that serum increases phagocytosis of carbon and that its effectiveness in this respect is destroyed by heating to 56°C. for 40 minutes.

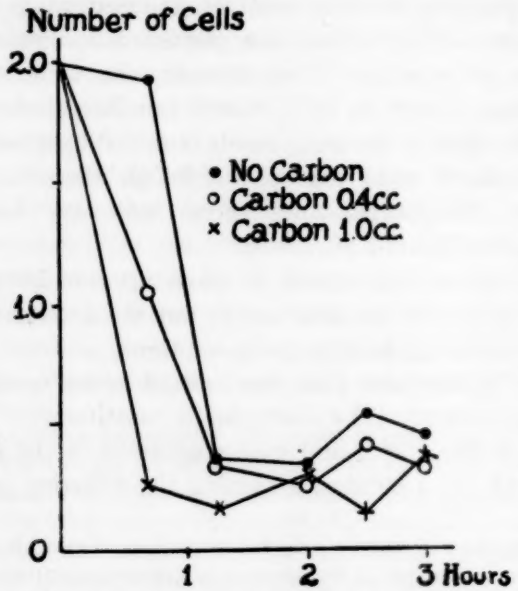

FIG. 6. The number of unclumped cells ($\times 10^4$ per mm.^{3}) plotted as ordinates against time in hours as abscissæ. Curves show that the cells clump more rapidly in presence of 3.1 and 1.25 $\times 10^4$ particles of carbon per mm.^{3} The same effect was observed in two other unpublished experiments in which counts of the cells were made.

ingested for which proteins (as ovalbumin) other than serum proteins may be used.

When cells are incubated in dilute serum with carbon they agglutinate more rapidly than when carbon is omitted. A typical experiment is plotted in Fig. 6. These data were taken from an early experiment when the cells from the peritoneal exudate were not washed in sodium chloride. Under these conditions agglutination of the cells occurred to a considerable extent, since it was impossible to add enough sodium citrate to prevent it without preventing phagocytosis as well. The degree of agglutination in this figure is, therefore, not characteristic of the other experiments in this paper.

Three curves are plotted showing the number of cells still unclumped without carbon, and with the addition of two concentrations of carbon. More cells are always free in the control. The obvious conclusion is that the process of ingesting carbon makes the cells more sticky so that they agglutinate more readily.

The result of the more rapid agglutination of cells in the presence of carbon is that the percentage of cells containing carbon is higher for the clumped cells than for the unclumped cells. This constitutes, therefore, a serious objection to the use of the percentage of cells containing carbon as a measure of phagocytosis (8). The reaction may appear to have come to a standstill when the aggregation of carbon-containing cells is merely keeping pace with the ingestion. Kite and Wherry (9) have also observed that clumped cells contain more bacteria than unclumped cells. This might be due, however, to the greater velocity of clumps of cells, the velocity increasing roughly as the two-thirds power of the number of cells in the clump. Neufeld and Rimpau (10), however, reported in 1905, from well controlled experiments, that phagocytizing leucocytes clump more rapidly than control leucocytes where phagocytosis is prevented by omission from the mixture of either the immune serum or the bacteria. This seems, therefore, to be a general phenomenon.

In experiments with unwashed cells the behavior of the cells is interesting though the results are worthless as accurate measures of phagocytosis. A typical experiment is plotted in Figs. 7 and 8. In Fig. 7 the number of cells still unclumped is plotted as ordinates against time as abscissæ. The percentage of these cells which con-

tained carbon is plotted as ordinates in Fig. 8. In 26 per cent serum nearly all the cells had clumped up in 15 minutes, after which they began to creep out of the clumps until at the end of 4 hours nearly one-half the original number was again free. In pure 0.9 per cent sodium chloride (control) there is least clumping and least phagocytosis. Similarly, in 26 per cent serum where clumping is most

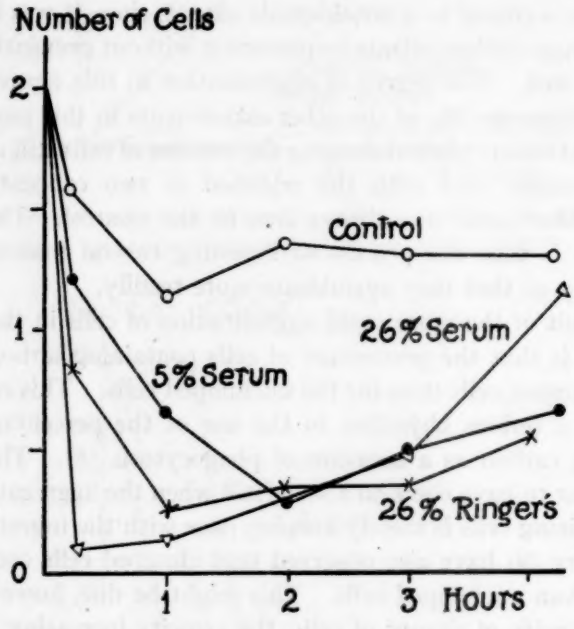

FIG. 7. Ordinates and abscissæ as in Fig. 6. Curves illustrate the effect of serum and an equivalent concentration of Ringer's solution on the clumping of leucocytes. After the first rapid agglutination, the cells begin to creep out of the clumps again.

rapid, phagocytosis is also most rapid. Part of the effect of serum is due to its calcium, since the same amount of Ringer's solution also increases both phagocytosis and clumping, though to a lesser degree. The small percentage of free cells containing carbon at the end of the 1st hour (Fig. 8) is probably due to the more rapid clumping of such cells, as already described (Fig. 6).

In general it was found in these experiments that anything which increased clumping also increased phagocytosis and *vice versa*. Thus, the higher the concentration of sodium citrate, the less the phagocytosis and the less the clumping.[3] With washed cells from which all the fibrinogen has been removed, the number of free cells shows only a gradual decrease during the experiment.

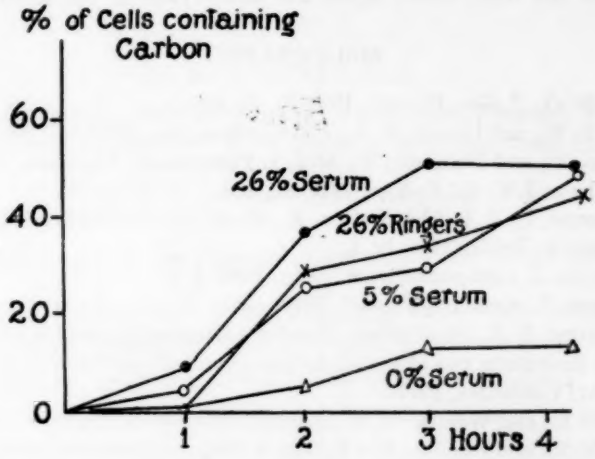

FIG. 8. Ordinates represent the per cent of the unclumped cells counted in Fig. 7, which contained carbon. Curves show that serum which accelerates clumping cells also accelerates phagocytosis. The similar effect of Ringer's solution shows that the calcium in serum is partly responsible for its effect.

SUMMARY.

1. By measurements of the diameter and velocity of leucocytes and of the particles in two carbon suspensions, the relative rates of ingestion of the two suspensions by the leucocytes are predicted and the predictions verified experimentally.

2. The results indicate that 4.7μ particles of carbon are ingested as readily as 3.2μ particles. The more rapid *apparent* rate of ingestion of the 4.7μ particles is due to their greater *availability* rather than the greater *capability* of the leucocytes.

[3] McJunkin (McJunkin, F. A., *Arch. Int. Med.*, 1918, xxi, 59) has used the ability of white blood corpuscles to ingest carbon in varying concentrations of sodium citrate as a means of classification.

3. There is almost no phagocytosis of carbon in absence of serum or in heated serum.

4. The clumping of unwashed leucocytes is accelerated by serum and by the ingestion of carbon.

5. The available evidence indicates that the phagocytosis of bacteria does not follow the law for a monomolecular reaction, possibly because of the toxic effect upon the leucocytes of bacterial extracts.

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AN INVESTIGATION INTO THE CAUSE OF THE SPONTANEOUS AGGREGATION OF FLAGELLATES AND INTO THE REACTIONS OF FLAGELLATES TO DISSOLVED OXYGEN.

PART I.

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The Phenomena of Spontaneous Aggregation and Band Formation.

It is well known that many flagellate and ciliate Protista form spontaneous aggregations. That is, when a drop of water containing the organisms is mounted on a slide for examination under the microscope the flagellates or ciliates are frequently seen to collect into clumps or masses. The center of such a collection may be a piece of some solid present in the water, or there may be no such visible focus. It is the second of the two cases that is here termed spontaneous aggregation, and the present investigation was undertaken with the object of finding out the cause of this phenomenon. The discovery of the cause then led on to the more general question of the relation of the organisms to varying amounts of dissolved oxygen.

Throughout the investigation the same species of flagellate was used; namely, *Bodo sulcatus*. It was originally described by Mereschkowsky.[1] For the present investigation the flagellate was obtained by taking grass from the garden behind the laboratory at Plymouth and steeping it in tap water. The best material was obtained from 6 day old cultures. The flagellates were then most abundant without being too much mixed with other organisms. In cultures older than 6 days there were too many bacteria present and, still later on, too many ciliates.

[1] Mereschkowsky, C., *Arch. mikr. Anat.*, 1879, xvi, 153.

The phenomenon of spontaneous aggregation is best studied as follows: A square cover-glass supported by wax feet at its four corners, or, better still, by short pieces of glass rod previously cemented to the corners, is placed on a dry slide. Some liquid from a *Bodo* culture is then run in from a pipette beneath the cover-glass until it just fills the space between the latter and the slide. At first the flagellates are evenly scattered throughout the preparation (Fig. 1) but they do not remain so for an indefinite time. At the end of an

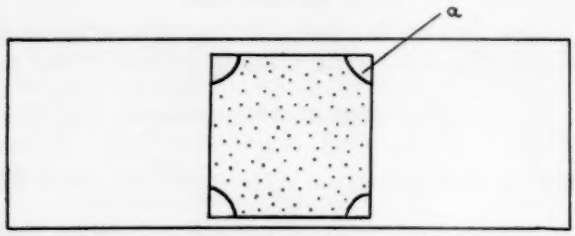

FIG. 1. Slide and supported cover-glass at beginning of an experiment, showing flagellates evenly scattered through the liquid beneath the cover-glass. In all diagrams the density of the dots represents the density of distribution of the flagellates. *a*, wax support to cover-glass. Figs. 1 to 5 are successive stages of one experiment.

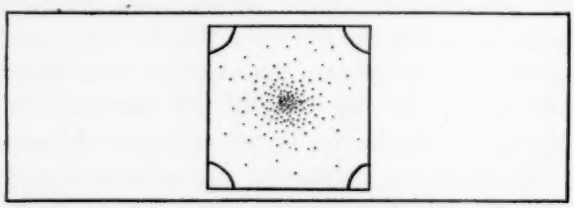

FIG. 2. Formation of central aggregation of flagellates.

interval which may be 2 minutes or 2 hours they begin to collect into one or more groups towards the center of the cover-glass, the size of these aggregations increasing until they contain most of the *Bodo* present in the liquid (Fig. 2). The flagellates in the aggregations are in intense movement. There is no solid body forming the center of a collection and indeed the presence of any such object is purposely avoided by filtering the suspension of *Bodo* through fine bolting-silk before making the preparation. Under a square cover-glass measuring $\frac{7}{8}$ inch $\times \frac{7}{8}$ inch, which was the size used in the

experiments, one central aggregation is usually formed but sometimes there are several, always near the center.

After an aggregation has been in existence for a short time a clear space free from flagellates appears at its center. The central space enlarges until the flagellates come to lie in a circular band around it, this band being easily visible to the naked eye (Fig. 3). The increase in size of the clear area goes on steadily, and as the band of flagellates surrounding it approaches the edges of the cover-slip the sides of the band become flattened (Fig. 4) and then, still enlarging, the band gradually comes to form a square, the sides of which are parallel to

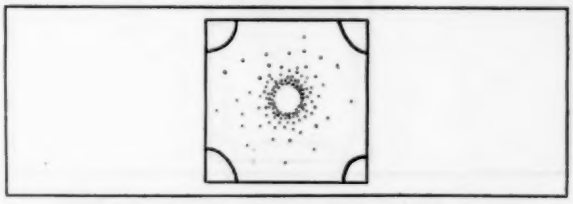

FIG. 3. Appearance of region free from flagellates in the center of the aggregation.

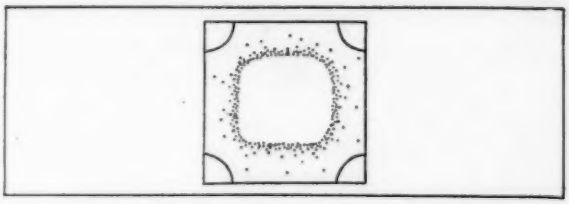

FIG. 4. Circular band of flagellates becoming square.

the sides of the cover-glass. When this square-shaped band of flagellates has reached a certain distance from the edges of the preparation, it becomes stationary, the central clear area no longer increasing in size (Fig. 5). The distance of the final position of the band from the edge of the cover-slip depends on the height of the latter above the slide; the band comes to lie the nearer to the edge the lower the cover-slip. The great majority of the flagellates present in the preparation are in the band, but nevertheless there are always a few swimming in the region between the band and the edge of the cover-glass.

A grain of sand lying in the path of the band of *Bodo* as it advances from the center towards the edges of the preparation has no effect on the band: the latter approaches and passes the grain of sand without being deflected. An air bubble, however, keeps the band at a distance from it. If the bubble lies sufficiently far in from the edge of the cover-glass the advancing band of flagellates becomes bent inwards to form a bay enclosing the bubble (Fig. 6). As the main band continues to move outwards the horns of the bay meet so that an inner ring of flagellates is left behind surrounding the bubble

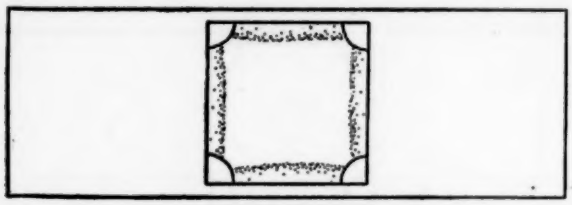

FIG. 5. Equilibrium position of flagellate band.

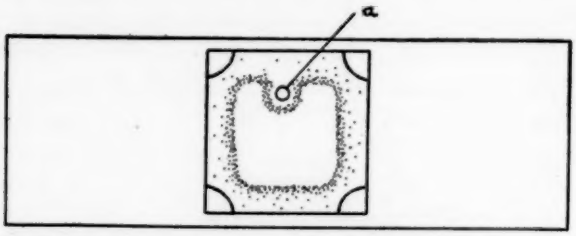

FIG. 6. Air bubble keeping back an advancing flagellate band. *a*, air bubble. Figs. 6, 7, and 8 are successive stages of one experiment.

(Fig. 7). The bubble, then, behaves to the band just as the water-air surface at the edges of the preparation does: it keeps the flagellate band at a certain distance from it. But there is this difference between the two cases, that whereas the main band comes to a halt and remains stationary at a certain distance inside the edges of the preparation, the band encircling the bubble slowly approaches the latter. This continues until the flagellates come into contact with the surface of the bubble itself (Fig. 8), where they remain for a short time and then dissipate, swimming out to join the main band.

If a preparation such as has been described, in which the square-shaped band of *Bodo* is established with its sides parallel to the edges of the cover-glass, is kept in a moist chamber and examined again on the following day, it will be found that the band has retreated somewhat from the edges of the cover-slip. On the following day again the band will be further in still and will have taken on a circular instead of a square form. Later on all the flagellates will be clumped in one mass at the center of the slide, after which they will gradually dissipate to become evenly scattered throughout the liquid again. In fact the *Bodo* band goes through the same series of changes which

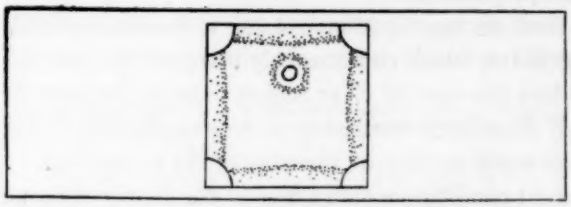

FIG. 7. Main flagellate band reaches its equilibrium position having left behind an inner ring surrounding air bubble.

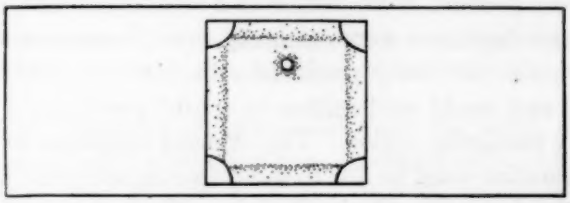

FIG. 8. Flagellates around air bubble advance to its surface.

it originally underwent in its formation but in the reverse order. Through all the series of changes the organisms continue in full motile activity. The retiral of the band from the outside may be hastened by placing the slide in an ice chest. In the course of a few hours only, the square band will have become a circular band situated near the center of the preparation. The swimming activity of the *Bodo* is, incidentally, not noticeably decreased by the low temperature. If, on the other hand, the moist chamber containing the preparation is kept at a higher temperature than that of the laboratory, the band retires from the edges much more slowly, if at

all. Furthermore, a flagellate band which has been caused to retire inwards by keeping the preparation at a low temperature will move outwards again as soon as the slide is replaced in the higher temperature of the laboratory.

The Cause of Aggregation and Band Formation.

In seeking for the causes of these phenomena, there are three questions to be asked.

1. What is the cause of the aggregation of the flagellates at the center of the preparation?

2. Why does an aggregation become a band surrounding a region clear of flagellates, which continuously increases in size?

3. Why does the central clear region cease to increase in size when the band of flagellates bordering it has reached a certain distance inside the air-water surface at the edge of the cover-slip?

The different condition which arises at the center of the preparation, attracting thither the flagellates, must be due to some substance or substances produced by the organisms themselves. It cannot be any excretory product of the nature of a solid in solution or a liquid, for, since the flagellates were originally evenly scattered throughout the preparation, the newly produced substance too would be evenly distributed and would tend neither to attract nor to repel the organisms in any particular region. The changed condition in the center of the preparation must be due to some volatile substance in solution, which at the free edges of the liquid is effecting an exchange with the atmosphere, either going into or out of solution. The only gases in solution which would be changed in amount by the living organisms are oxygen and carbon dioxide; the amount of the former present in solution in the water must continuously decrease and the amount of the latter increase. These changes in the concentrations of dissolved oxygen and carbon dioxide will take place more rapidly in the central region of the liquid than at its edges; for at the edges the oxygen used up by the flagellates will be replaced from the atmosphere, while the carbon dioxide produced by them in this region will go out of solution into the atmosphere as soon as its tension in solution rises above the value corresponding to its partial pressure in the

atmosphere. In the center of the preparation no exchange with the air can take place, so that here the concentration of dissolved oxygen will decrease and that of carbon dioxide increase most rapidly.

It seems probable, then, that the flagellates collect in the central region because they are attracted either (*a*) into a region of higher hydrogen ion concentration, or (*b*) into one where the concentration of dissolved oxygen is lower. Which of these alternative explanations is the correct one? It was attempted to answer this question by isolating the two possible causes and allowing each to act separately.

To test suggestion (*a*) a long cover-glass was supported over a slide by wax feet placed beneath its four corners. Some filtered suspension of *Bodo* was then let in under the cover-glass from one end, so as not to fill completely the space between the cover-glass

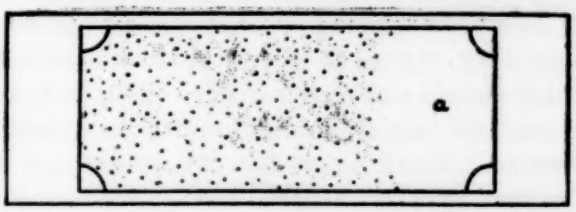

FIG. 9. Even distribution of flagellates beneath a long cover-glass unaffected by the introduction of carbonic acid at one end. *a*, carbonic acid.

and the slide. Immediately afterward from the other end of the cover-glass some water which had been saturated with carbon dioxide was let in (Fig. 9). If such a solution had an attractive influence on *Bodo*, the flagellates would have collected in the region where the two liquids merged. This they did not do.

To test suggestion (*b*) a similar preparation was made but in place of carbonic acid, reduced indigocarmine was introduced beneath one end of the cover-slip. Indigocarmine (sodium sulfindigoate) was reduced in the absence of oxygen (in a stoppered bottle) by a solution of 1 per cent glucose containing 1 per cent caustic potash. By this means the yellow leuco base is formed. As much of a concentrated solution of indigocarmine was used as the glucose would turn from blue to yellow in 1 hour in the stoppered bottle. In the presence of oxygen the yellow leuco base reoxidizes instantane-

ously to blue indigocarmine. This takes place either when the leucobase is exposed to the air or when it comes into contact with water containing oxygen in solution. In our experiment under the cover-glass the dissolved oxygen was abstracted from a zone of the *Bodo* suspension bordering the drop of reduced indigocarmine. It was found that all the flagellates from the neighboring region of the suspension collected rapidly in this zone, forming there a crowded band (Fig. 10). Thus the *Bodo* are attracted into a region whence the dissolved oxygen has been removed. As control tests, indigocarmine alone, glucose alone, and caustic potash alone were substituted for the mixture of the three but in no case was there any aggregation of *Bodo*. This experiment was repeated with precisely the same result using in place of the reduced indigocarmine either

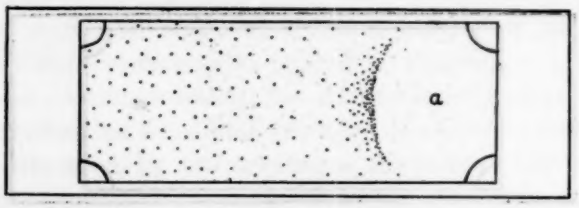

FIG. 10. Flagellates beneath a long cover-glass attracted by an oxygen absorber introduced at one end. *a*, oxygen absorber.

an alkaline solution of pyrogallic acid or of hematoxylin. Both of these solutions take up oxygen rapidly. In the case of the hematoxylin the best procedure was found to be to color the *Bodo* suspension lightly with the dye, which does not injure the flagellates, and then to introduce some of this suspension under one end of a long, supported cover-slip. Under the other end 0.1 N NaOH was let in. In the zone where the two liquids meet, the hematoxylin rapidly oxidizes in the presence of the alkali, abstracting oxygen from solution in the water. The flagellates in the neighboring region collected in a crowded band in this zone of reduced oxygen concentration. Controls in which the alkali was replaced by water and in which the hematoxylin was omitted gave no aggregation of flagellates.

In this way it can be demonstrated that the flagellates will collect into a region where the concentration of dissolved oxygen is reduced

but that they will not react to a region of greater hydrogen ion concentration caused by dissolved carbon dioxide. The aggregation then in the center of the liquid beneath a cover-slip must be due to the attractive influence of this central region where the respiratory activity of the organisms has reduced the amount of dissolved oxygen.

The second question—why does an aggregation become a ring surrounding an ever growing clear area—has now to be answered. Do the *Bodo* leave the central area (*a*) because the concentration of dissolved oxygen has fallen here below an optimum value for them or (*b*) because the hydrogen ion concentration has increased too much?

If (*b*) is the cause, the flagellates should leave the central area sooner—that is, they should form a ring sooner—in a preparation which was originally more acid than in one originally less acid. For in the former the critical concentration of H ions which would drive out the flagellates would be arrived at earlier. This test was made. One sample of a culture of *Bodo* was given a concentration of H ions such that when tested with rosolic acid it gave the same yellow color as did tap water saturated with carbon dioxide. A second sample from the same *Bodo* culture was given a concentration of H ions showing a pink with rosolic acid. These changes in the H ion concentrations of the two samples had no effect on the activity of the flagellates as judged under the microscope. If the cause of the formation and spreading of the band is the accumulation of carbonic acid at the center of the liquid, the occurrence must take place sooner in a preparation made from the first sample than in one made from the second. The experiment showed, however, that the band was formed and spread simultaneously in the two preparations.

It must be mentioned here that in this and all other experiments when the times of aggregation or band formation were to be compared in two preparations, the cover-glasses were supported by short pieces of thin glass rod cemented to their corners, not by wax feet. By using pieces of glass having the same thickness it was ensured that the cover-glasses were at the same height above the slides. This is necessary since the height of the cover-glass influences the rate of aggregation and the distance of the equilibrium position of the band from the edge.

It is not carbonic acid, then, that drives out the *Bodo* from the center and we must conclude that, while the organisms collect into a region where the oxygen concentration is lower than the saturation concentration for the atmospheric partial pressure, yet when the oxygen in this region falls below a certain limiting value the organisms are forced to move away again. They remain in a band surrounding the central area of lowest oxygen concentration, this band representing the optimum concentration of oxygen for them. It lies between the central exhausted region and the outside liquid into which oxygen continually dissolves from the air to replace that used up. Further, since in the crowded band the *Bodo* consume the available oxygen more rapidly than it can be replaced from outside, the size of the central area of lowest oxygen concentration continually increases, forcing the band to approach nearer and nearer to the edges of the cover-slip.

The fact that the *Bodo* band is a zone of optimum oxygen concentration at once gives the clue to the third question originally asked—why does the advancing band cease to advance when it has reached a certain distance from the edges of the cover-glass? As the band advances towards the water-air surface at the edges of the preparation it must eventually reach a position where there is a state of equilibrium between the oxygen used up by the flagellates and that diffusing inwards from the edges. At this point the band will remain stationary.

That this inward diffusion of oxygen is really the factor which controls the distance of the band of flagellates from the edge can be shown very simply as follows. A preparation with a *Bodo* band which has become stationary is placed in a gas chamber on the stage of a microscope and oxygen is passed through the chamber. Almost immediately the flagellate band commences to retire inwards towards the center of the liquid under the cover-slip. It gets gradually less square and more circular until it forms a small ring and finally becomes one mass of flagellates at the center of the preparation. When now a stream of hydrogen is passed through the gas chamber in place of the oxygen, the band reforms and slowly but continuously increases in circumference. In the first case the oxygen dissolving in the edges of the liquid and diffusing inwards causes the position of opti-

mum oxygen concentration for the flagellates to move inwards. In an atmosphere of hydrogen, on the other hand, the oxygen goes out of solution again around the free edges of the liquid and the position of optimum oxygen concentration for the flagellates again moves outwards.

Thus oxygen is the controlling factor in the position which the flagellates take up: they collect into regions where the concentration of dissolved oxygen is an optimum for them. The behavior of the advancing band in the presence of an air bubble now receives its explanation. The bubble acts at first like the edges of the preparation, the water immediately around the bubble being saturated with oxygen at the atmosphere partial pressure of the gas, so that the flagellates are unable to move right up to its surface. But as the flagellates are continually taking up oxygen, the amount of the latter present in the bubble gradually becomes exhausted. The flagellates are thus enabled to approach closer to the bubble until they touch its surface, where they remain for a short time until nearly all the oxygen in the bubble has gone into solution and been consumed by them. Then the oxygen concentration at the surface of the bubble falls below the optimum for the *Bodo* and they leave the region altogether.

This phenomenon can be imitated as follows. A cover-slip with wax legs is placed on a slide and some of the indigocarmine-glucose-caustic potash mixture let in beneath it with a pipette. The central area of the preparation soon becomes yellow, the indigocarmine being reduced here in the absence of oxygen. This yellow area is square with its sides parallel to the edges of the cover-glass. It is surrounded by a purple band, the region between this purple band and the edge of the liquid being blue. The purple band corresponds exactly to the flagellate band. It is the region of equilibrium between oxygen consumed at the center and oxygen diffusing in from the edge. Any air bubble in the yellow area is surrounded at first by a narrow blue ring, separated from the yellow by a circular purple band. The purple band approaches slowly but continuously to the bubble until it lies on the surface of the latter and then it gradually disappears. This occurs when all the oxygen of the bubble has gone into solution and been used up. We have here an exact parallel to the behavior of a *Bodo* band surrounding an air bubble.

We must now ask, what is the cause of the gradual slow retiral of a *Bodo* band from the edges of a preparation when left over night at room temperature, of its more rapid retiral in the cold, and of its spreading out again when replaced at a higher temperature? These phenomena also can be imitated with an indigocarmine preparation such as has just been described. When the slide with its yellow central region separated from the blue border by a square purple band, the sides of which are parallel to the edges of the cover-glass, is placed in the ice chest the purple band contracts. It retires towards the center of the solution, the corners of the square becoming rounded until it assumes the form of a ring. When the preparation is now replaced at a higher temperature the purple ring expands again, becoming square as it nears the edges of the cover-glass. When left over night in a moist chamber at room temperature the purple band recedes slowly from the edges of the liquid. Now this behavior of the purple band of partially reduced indigocarmine is due to the different solubilities of oxygen at different temperatures. On the ice more oxygen goes into solution and drives the purple band inwards. When the preparation is warmed some of the oxygen goes out of solution and the purple band approaches the edges again. Left at room temperature the concentration of dissolved oxygen at the edges of the preparation gradually rises because the water was not originally saturated with oxygen, so that the band retires slowly inwards. The cause of the similar behavior of a *Bodo* band under the same conditions must be precisely the same. The band of flagellates moves to a position nearer to or further from the edge according to the lesser or greater amount of oxygen going into solution at the different temperatures. An alternative explanation is that the *Bodo* band retires inwards at a low temperature or when left for some time because under these circumstances the flagellates change their oxygen optimum. The suggestion, however, becomes very improbable in view of the parallel behavior of the indigocarmine band, the cause of which is known.

Confirmatory Facts.

There are several facts and experiments which strikingly confirm the conclusion that the concentration of dissolved oxygen is the con-

trolling factor in the behavior of the flagellates. Of these I shall mention three. They are: (1) the effect of the original oxygen content of the *Bodo* suspension on the rate of band formation; (2) the reduction of oxyhemoglobin by *Bodo*; and (3) the effect of a green plant in sunlight on the band.

To show the effect of the oxygen content of the suspension of flagellates on the rate of aggregation and band formation two preparations were made on slides in the manner already described. In the first preparation the liquid was let in beneath the supported cover-glass immediately after having been pipetted out of the culture jar. For the second preparation a pipetteful of liquid was taken from the same culture jar, placed in a petri dish, and exposed to the air for a short time before being let in under the cover-glass. A *Bodo* culture always contains less oxygen in solution than plain water in a similar jar at the same temperature. This is of course due to the fact that the flagellates are continually absorbing oxygen in respiration. Consequently the suspension of *Bodo* which had been exposed to the air in the petri dish had acquired a greater oxygen content than that taken directly from the culture jar. It was found that the aggregation of flagellates was much slower in the aerated than in the non-aerated preparation and that the central clear area became established later in the former.

It was to be expected that the central clear area bordered by the band of flagellates would become established later in the aerated preparation. Here it must take longer for the organisms to consume sufficient oxygen to make the central region untenable for them. But it is not at first obvious why the central aggregation should form later in the aerated preparation. It would be expected, rather, that when in the presence of more oxygen than the optimum the flagellates would migrate from any region of higher into any region of lower oxygen concentration. Now a region of *relatively* lower oxygen concentration due to the respiration of the flagellates must arise as soon in the center of the aerated as of the non-aerated preparation; nevertheless, in the former the organisms are not attracted so soon towards the center as they are in the latter. It seems thus that when the liquid is well aerated the flagellates are not sensible to a region of lower oxygen concentration: it is not until the oxygen content has been

reduced by a certain amount throughout the slide that the *Bodo* feel the attraction of the central region. They are thus only sensible to this influence when in the presence of an oxygen concentration closely approaching the optimum for them.

This was confirmed by an experiment with an oxygen absorber such as reduced indigocarmine or pyrogallic acid. Two slides were prepared with long cover-slips supported by pieces of glass rod. Under one cover-slip was run in some *Bodo* suspension taken straight from the culture jar and under the other some of the same culture which had been aerated by exposure in a petri dish. The liquid was not allowed in either case completely to fill the place beneath the cover-slip, but a space was left at one end into which the oxygen absorber was to be run. When this had been done, it was seen that the flagellates in the aerated preparation collected much later into the region of reduced oxygen concentration next to the indigocarmine than they did in the non-aerated preparation. In fact whereas in the non-aerated liquid the *Bodo* moved at once into the region next the oxygen absorber, in the aerated liquid they did not do this until by their own respiration they had reduced the oxygen content of the general suspension to a point approaching the optimum for them. The flagellates thus gave no response to lowered oxygen concentration when in the presence of an amount of oxygen much above their optimum.

It was mentioned at the commencement of this account that in a normal cover-slip preparation the *Bodo* ring may become established in 2 minutes or it may take 2 hours to form. A series of experiments demonstrated that this great difference in time is due to different initial oxygen contents of the suspensions. If the suspension to be used is exposed to the air for some time during the preparatory filtering operation it will have a higher oxygen content than liquid taken straight from the culture jar, for the oxygen concentration in the cultures is always considerably lower than the saturation concentration under atmospheric partial pressure, and therefore in the filtering through bolting-silk oxygen is absorbed from the air. Aggregation and ring formation always take place later in a suspension that has been exposed to the air than in one that has not.

The following is an account of four experiments which illustrate this point. The same *Bodo* culture was used throughout. In each experiment two preparations were made; (*a*) with *Bodo* suspension taken straight from the culture jar, and (*b*) with a suspension previously aerated. In Experiment 1 the aeration was done by pouring from one watch-glass to another for 15 seconds. The times taken for the band of flagellates to take up its stationary position were; (*a*) 11 minutes, and (*b*) 45 minutes. In Experiment 2 the aeration was done in the same way and the times were; (*a*) 4 minutes, and (*b*) 46 minutes. In Experiment 3 the aeration was performed by leaving a small quantity of the liquid in a watch-glass for 56 minutes at 18°C., the laboratory temperature being 23°C. The times were; (*a*) 2 minutes, and (*b*) 56 minutes. In Experiment 4 for the preparation of Slide (*a*) 10 cc. of *Bodo* suspension were kept in a watch-glass for ½ hour at 26° and for (*b*) 10 cc. were similarly treated at 17°. There was no noticeable difference in activity of the flagellates at the two temperatures. As soon as the liquids were pipetted under the cover-glasses their temperatures became identical and equal to that of the laboratory so that the different times taken for the bands to become established must have been due to the different quantities of oxygen which had gone into solution at the two temperatures. The times were; (*a*) 2½ minutes, and (*b*) 14 minutes.

The consumption of oxygen in the center of developing *Bodo* rings can be demonstrated in preparations containing hemoglobin. For this purpose a pipetteful of liquid from a *Bodo* culture is placed in a watch-glass and one or two drops of blood from a pricked finger are mixed with it. The blood becomes laked. It in no way interferes with the activity of the flagellates. If now a preparation is made in the usual way beneath a supported cover-slip, no sooner does the central aggregation of flagellates become a ring than the bluish color of the region surrounded by the ring shows that it contains reduced hemoglobin. The blue color here is in marked contrast to the scarlet of the oxyhemoglobin outside the ring of flagellates, and the distribution of hemoglobin and oxyhemoglobin is easily verified with the microspectroscope. Thus after the flagellates have used up the available free oxygen dissolved in the water, they extract that which is bound in the oxyhemoglobin. When this too is exhausted at the

center of the slide, a circular band of flagellates is formed surrounding a region of reduced hemoglobin. As would be expected, aggregation and band formation take place much more slowly in the presence of oxyhemoglobin than in plain water for much more oxygen is available.

In a typical case two preparations were made simultaneously from the same culture; (*a*) without blood, and (*b*) with blood. During the mixing of (*b*) with blood, (*a*) was exposed to the air in exactly the same way as (*b*). The times for the ring to become established in its stationary position were; (*a*) 1 hour, 15 minutes, and (*b*) 3 hours, 40 minutes.

In the preparations containing hemoglobin the circular *Bodo* zone surrounding the central clear area, when once formed, grows in the usual way, becomes square, and takes up its stationary position some

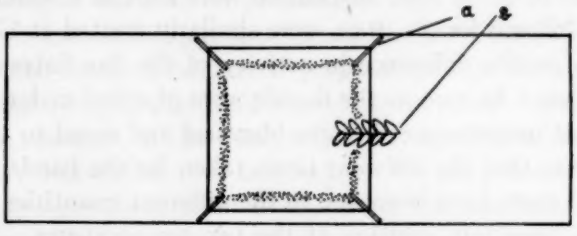

FIG. 11. Preparation kept in darkness with flagellate band in equilibrium position interrupted by a piece of moss. *a*, glass rod supporting cover-glass; *b*, moss.

distance inside the edge of the cover-slip. The whole central area within the band contains reduced hemoglobin while the edges outside it show the bright scarlet of oxyhemoglobin. The flagellate band itself lies just within the region of reduced hemoglobin.

A demonstration of the effect of oxygen on the equilibrium position of the *Bodo* band can be arranged as follows. Some suspension of *Bodo* is let in with a pipette under a cover-glass supported at its corners, and a piece of some aquatic green plant, such as a frond of moss, is pushed into the liquid from the middle of one side of the cover-glass. The slide is placed in the dark and when the band of flagellates becomes established in its stationary position it will cut straight across the moss (Fig. 11). The preparation is now exposed to diffuse sunlight, so that oxygen is produced by the plant in photosynthesis.

The edges of the flagellate band which touched the moss on either side immediately move back from it and bend inwards (Fig. 12) and in a few minutes the band has reformed with an indentation to include the moss (Fig. 13). Replaced in the dark the band straightens

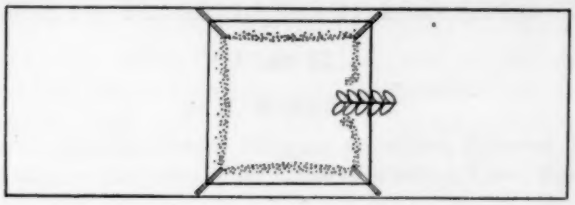

FIG. 12. Bending inward of the ends of the flagellate band in contact with the moss when the preparation shown in Fig. 11 is brought from darkness into daylight.

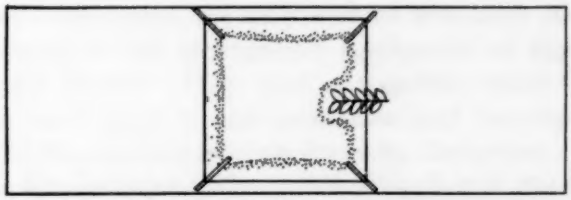

FIG. 13. Reformation of the flagellate band inside the moss after the preparation shown in Figs. 11 and 12 has been in daylight for a short time.

out again to its original position as the extra oxygen which was produced by the plant in the light is consumed by the flagellates. The two ends of the band in touch with either side of the moss bend slightly outwards towards the edge of the slide (Fig. 11) because the moss is now absorbing oxygen.

AN INVESTIGATION INTO THE CAUSE OF THE SPONTANEOUS AGGREGATION OF FLAGELLATES AND INTO THE REACTIONS OF FLAGELLATES TO DISSOLVED OXYGEN.

PART II.

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(Received for publication, September 21, 1920.)

Previous Work.

As far as I am aware, the only workers who have attempted to find the causes of the spontaneous aggregation of flagellates are Jennings and Moore.[1] They used a flagellate called *Chilomonas paramecium* and came to the conclusion that the organisms are attracted by the carbonic acid produced by themselves. Where by chance the flagellates are more crowded together in the liquid on a slide, more carbonic acid is produced than elsewhere and in consequence yet more flagellates are attracted into these regions. The experimental evidence on which they based their conclusion was as follows. They found: (1) that when a dilute mineral acid was let in under the cover-slip by means of a capillary pipette into a suspension of the flagellates the latter were attracted by the drop; and (2) when a bubble of carbon dioxide was introduced in the same manner the organisms crowded around it, whereas they did not collect around an air bubble. Therefore, Jennings and Moore concluded, in the formation of spontaneous collections the flagellates are attracted to regions which become more acid than the remainder of the liquid, through the accumulation of the carbonic acid produced by the individuals already there.

[1] Jennings, H. S., and Moore, E. M., Studies on reactions to stimuli in unicellular organisms, VIII. On the reactions of Infusoria to carbonic and other acids, with especial reference to the causes of the gatherings spontaneously formed, *Am. J. Physiol.*, 1902, vi, 233.

I have found that both the experimental data of these workers are correct, but that nevertheless they do not lead to their conclusion. I tried the effect of mineral acids on *Bodo* by letting the acid in under one end of a long, supported cover-slip, under which some *Bodo* suspension had just previously been introduced. In a zone parallel to the junction of the two liquids a band of flagellates rapidly accumulated. A series of such tests was made with acids of decreasing strengths and it was found that the reaction became feebler each time until a point was reached (at 0.0005 N HCl) when the *Bodo* no longer reacted to the acid at all. Nevertheless 0.0005 N HCl, tested with Congo red as indicator, is a considerably stronger acid than a saturated solution of CO_2 in water. Congo red is turned violet by 0.0005 N HCl whereas the scarlet color of this indicator in distilled water takes on a very slight bluish tinge only when the solution is saturated with CO_2.

Further it has already been shown (page 489) that the flagellates do not collect in the region next a drop of water saturated with CO_2 let in under the cover-slip. Jennings and Moore made no attempt to determine quantitatively the exact strength of acid solution to which these organisms react. In fact, we are dealing here with a phenomenon quite unconnected with spontaneous aggregation, a reaction of the flagellates to acids and the weakest effective acid is stronger than carbonic acid.

It is worthy of remark that the accumulation of a crowded zone of *Bodo* in the region in front of an acid introduced beneath the cover-slip is much more marked in aerated than in non-aerated suspensions, which is the reverse of the reaction of the flagellates to a region of reduced oxygen concentration. Indeed an acid as weak as 0.001 N HCl produces a slight collection in an aerated preparation only and none in one made by taking liquid straight from the *Bodo* culture jar. The crowded zone next the acid always dissipates before the subsequent commencement of aggregation and band formation of the flagellates in the region of low oxygen concentration.

With regard to the gas bubbles, it is true that flagellates collect around a carbon dioxide bubble and not around an air bubble, but it is equally true that flagellates collect around a hydrogen bubble. Jennings and Moore did not try the hydrogen bubble. It is evidently

to reduced oxygen concentration that the organisms are attracted both in the case of the carbon dioxide bubble and of the hydrogen bubble. Part of the oxygen in solution in the water diffuses into the gas bubble, where the partial pressure of oxygen is nil, and so a region of reduced oxygen concentration is established in the water around the bubble. The flagellates are attracted into this region just as they are attracted into a central region under the cover-glass where the oxygen concentration is lowered by their own respiration. Just as in the latter case the aggregation occurs sooner in a non-aerated than in an aerated preparation, so the response to a hydrogen bubble is found to be greater in a non-aerated suspension.

The flagellates collect immediately around a hydrogen bubble but the intensity of the aggregation diminishes with time, until after a certain interval there are no more *Bodo* around the bubble than in the general suspension. This is because the bubble has now absorbed from the water enough oxygen to be in equilibrium with the dissolved oxygen and there is no longer a region of low oxygen concentration in the water around the bubble. After this the continually decreasing oxygen tension in the water, due to the respiration of the flagellates, falls below that in the bubble and the flagellates move away from the latter which is now giving back oxygen to the water. The center of the preparation is then cleared of *Bodo*, the peripheral band formed in the usual way, and in this process the bubble acts as an air bubble, becoming surrounded by an inner ring of flagellates which gradually approaches it.

Thus the experiments with acids and with gas bubbles not only dispose of the view that spontaneous aggregations are due to positive chemotropism to acid produced by the respiration of the flagellates but they also strengthen the explanation of the phenomenon given above.

The Effects of Excess and of Absence of Oxygen.

There is a relation between the oxygen content of the *Bodo* culture media and the preference shown by the flagellates for an optimum oxygen concentration lower than the saturation concentration of the gas in water in contact with air. For a series of determinations showed that, in the grass infusions contained in upright unstoppered

glass jars in which the *Bodo* swarmed, the oxygen content was considerably lower than that in a similar vessel containing an equal volume of tap water.

A number of experiments was made to test the effects, if any, of excess and of absence of dissolved oxygen on *Bodo*. An ordinary cover-slip preparation was made and at the same time an equal amount of *Bodo* suspension was placed on a hollow-ground slide and exposed to the air in a moist chamber. 24 hours afterwards the usual square-shaped band of flagellates was present under the cover-glass, in the zone of optimum oxygen concentration, while on the uncovered slide the flagellates were evenly distributed throughout the drop. In spite of the oxygen concentration being above the optimum in the second preparation, the swimming activity of the individuals was alike in both. The fact that a large excess of oxygen has no effect either was demonstrated at the conclusion of a gas chamber experiment such as that described on page 492. A preparation with an established *Bodo* band was placed in the chamber and oxygen passed through the latter. The square band retired towards the center becoming a ring and then a single mass in the middle after which the flagellates dissipated to become evenly distributed through the slide. This dissipation always occurs when the oxygen concentration is more than a certain amount above the optimum; it was seen also in preparations left standing for some time (page 487). In the present instance the oxygen concentration must have been very greatly above the optimum since the gas chamber contained pure oxygen. The chamber was now closed and the preparation left over night in the oxygen atmosphere. 17 hours after the commencement of the experiment the flagellates were swimming in full activity. The preparation was then removed from the gas chamber and exposed to the air. In 3 hours and 10 minutes two aggregations had formed towards the center and in 10 hours and 10 minutes the square band was reestablished. Thus 17 hours in the presence of an oxygen concentration higher than they could ever encounter in nature had no inhibitory effect on the activity of the flagellates.

To test the effect of the complete absence of oxygen an experiment was devised as follows. An ordinary preparation was made of *Bodo* suspension beneath a supported cover-slip. This was then closed

along the four sides with vaseline so that the suspension was everywhere shut off from the air (Fig. 14). For comparison a second preparation was made which was vaselined along two of its four open edges. This was done to eliminate any possible injurious effect of the vaseline on the *Bodo*, which would now be the same on both slides. 24 hours afterwards almost all the flagellates in the sealed preparation were motionless, just a few swimming feebly or vibrating. In the open preparation there were normal bands of flagellates at a

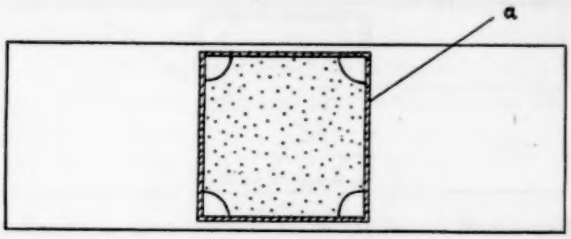

FIG. 14. Experiment to test the effect of the absence of oxygen on flagellates. Edges of the cover-glass were sealed with vaseline. *a*, vaseline.

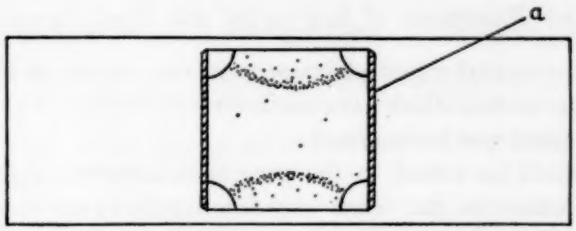

FIG. 15. Experiment to test the effect of the absence of oxygen on flagellates. Two sides of the cover-glass were sealed with vaseline. Distribution of flagellates after 24 hours. *a*, vaseline.

certain distance inside the free edges (Fig. 15). 48 hours from the start of the experiment the *Bodo* in the sealed slide were in the same motionless condition as on the previous day, while those in the open preparation were in full activity, the bands having retracted in the usual manner to form a ring near the center. A small aperture was now made in the vaseline wall surrounding the sealed preparation so that the air came into contact with the water of the suspension at this point. Instantaneously all the *Bodo* in the neighborhood of

the aperture commenced to swim actively and arranged themselves in a band at a certain distance inside the aperture (Fig. 16). This semicircular band gradually retreated inwards as the atmospheric oxygen diffused in. The effect of the absence of oxygen is thus not immediately injurious; it stills the motion of the flagellates but after 48 hours in the absence of oxygen they recover their full activity as soon as they have access to oxygen again.

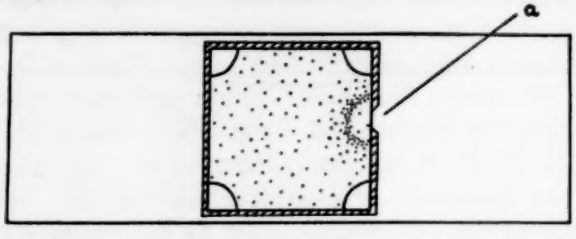

FIG. 16. Experiment to test the effect of the absence of oxygen on flagellates. Distribution of the flagellates in the preparation already shown in Fig. 14, when a hole was made in the vaseline wall at the end of 48 hours. *a*, aperture in vaseline.

Minor Phenomena of Aggregation and Band Formation.

There are several minor phenomena in the process of aggregation and band formation which have not been mentioned up to the present but which must now be described.

1. It should be added to the general description of aggregation and band formation that when very few flagellates are present in the liquid no central aggregation is formed, but after a certain time the flagellates simply leave the central region of the preparation and arrange themselves directly in the final stationary position of the band.

2. A vertical section of the *Bodo* band is not an upright wall but has the shape shown in Fig. 17. The upper part of the band is nearer to the center of the preparation than the lower part. Further, there are more flagellates present in the upper and lower regions, that is next the cover-glass and next the slide, than in the intermediate region. The latter is curved in section, the concavity being towards the edge of the preparation. The band assumes this shape as soon as it forms and keeps the same shape when in its final station-

ary position. The relative density of flagellates in the upper and lower parts of the band varies in different preparations, being sometimes more and sometimes less marked. The degree of slope of the section of the band also varies, being sometimes more and sometimes less steep. The consequence of the greater concentration of *Bodo* at top and bottom of the band and of the slope of the latter is that when the preparation is viewed from above the band appears to be double (Fig. 18). The outer and inner lines of this double band naturally appear the further separated from one another the higher the cover-glass is placed above the slide.

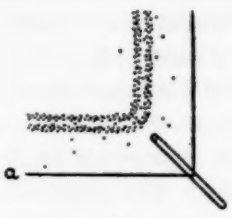

FIG. 18.

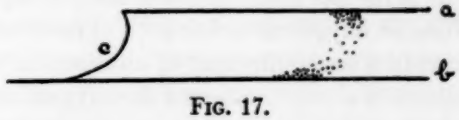

FIG. 17.

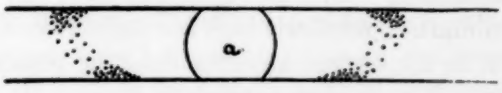

FIG. 19.

FIG. 17. Vertical section of a flagellate band. *a*, cover-glass; *b*, slide; *c*, water-air surface.

FIG. 18. Enlarged view of one corner of a square flagellate band. *a*, edge of cover-glass.

FIG. 19. Vertical section through an air bubble and a ring of flagellates surrounding it. *a*, air bubble.

The section of the band has a similar shape to that of the water-air surface at the edge of the cover-slip. This is shown in Fig. 17. It was thought at first that the form of the water-air surface might determine the form of the band, but this is not the case for a flagellate ring surrounding an air-bubble has the same sectional shape as the main band whereas the section of the water-air surface bordering the bubble is a symmetrical curve as shown in Fig. 19. The reason for the peculiar shape of the *Bodo* band remains undetermined. It is unknown why those individual flagellates which are negatively geotropic seem to have a lower optimum oxygen concentration than those which are positively geotropic. The possibility naturally arose that there were two species of *Bodo* present or two different stages in

the life history of *Bodo sulcatus*. To settle this, the flagellates were fixed after they had formed a band by introducing the fixative beneath the cover-slip through a capillary tube previously placed in position. Fixed in this way many individuals remained in position attached to the glass so that the cover-slip could be lifted off the slide and then the upper and lower flagellates stained and mounted separately. The flagellates attached to the cover-glass were found to be identical with those on the slide. We are thus dealing with a single species but the possibility remains that the positively and negatively geotropic individuals are in different stages of their life cycle.

3. A further point that has not yet been mentioned is the following. When an oxygen absorber such as reduced indigocarmine is introduced beneath a cover-slip next to a suspension of *Bodo*, a band of flagellates collects in a zone bordering the oxygen absorber in the manner already described. After this principal band has formed a subsidiary band containing relatively very few individuals makes its appearance nearer in to the oxygen absorber than the principal band. The flagellates in this inner band are close up beneath the cover-glass and move very sluggishly in striking contrast to the intense activity of those in the principal band. When the latter moves away to take up its definitive position just inside the edges of the cover-slip, the inner band remains for some time in place and then gradually the individuals composing it dissipate to join the principal band.

The reason for this inner band may be that a few individuals have a lower oxygen optimum than the rest. This explanation seems unlikely, however, because the inner band is not a permanent one; after a certain time it dissipates. More probably some individuals chance to swim into the region of lowest oxygen concentration and are trapped there because, as was shown on page 504, the effect of absence of oxygen is to inhibit swimming activity. This suggestion is supported by the fact that in ordinary spontaneous band formation an inner band is best developed in those preparations in which the central region is cleared most rapidly. Here more individuals would be left behind, trapped in the region of least oxygen.

4. There are of course many kinds of bacteria present in the *Bodo* cultures. In the preparations on slides many of the bacteria arrange themselves in zones of optimum oxygen concentration in the manner

originally described by Beyerinck.[2] The bacteria, however, do not commence by forming obvious central aggregations which then become converted into rings. This is, perhaps, because they are less motile than the flagellates: they collect more slowly than the latter into their zones of optimum oxygen.

Now when a bacterial optimum position coincides with that of *Bodo*, there is frequently an antagonism between the two organisms, the bacteria preventing the flagellates from taking up their normal position. In particular, there were frequently present certain cocci the optimum position for which was situated at the middle depth of the *Bodo* band and certain bacilli whose optimum coincided with the lower and outer portion of the *Bodo* band. When a preparation was made from a culture in which these bacteria were present in quantity the *Bodo* were excluded from the middle and lower parts of their zone and all forced to occupy the upper and inner part. The bacteria took up their position more slowly than the flagellates, consequently when the band first formed the flagellates occupied both upper and lower regions, as shown in Fig. 17, but when the bacteria arrived they drove the flagellates out of the lower region. When a preparation having a *Bodo* band with these bacteria in its lower part and a piece of moss cutting across the band as described on page 498 is removed from darkness to light, all the flagellates and bacteria move inwards to take up a new position, as shown in Figs. 11, 12, and 13 (Part I). But the *Bodo* move back much more rapidly than the bacteria and, arriving first at the new position of optimum oxygen, are able to place themselves in a band extending in depth from cover-glass to slide. Later on the bacteria arrive and drive out the flagellates from the lower and middle parts of their zone.

SUMMARY.

Spontaneous aggregations of flagellates are formed under the cover-glass because the organisms are attracted to and remain in regions where the concentration of dissolved oxygen is less than the saturation concentration under atmospheric partial pressure. These regions of

[2] Beyerinck, M. W., Über Atmungsfiguren beweglicher Bakterien, *Centr. Bakt.*, 1893, xiv, 827.

lessened oxygen content arise towards the center of the liquid beneath the cover-glass, owing to the oxygen consumed by the flagellates in respiration not being replaced here by the solution of atmospheric oxygen, as it is along the edges of the liquid. The flagellates, however, are insensitive to the attraction of regions of lessened oxygen concentration when the oxygen concentration throughout the liquid is above a certain value. Therefore, for the aggregations to form, either the initial concentration of dissolved oxygen must be below this limiting value, or an interval of time must first elapse after the making of the preparation until the respiration of the organisms has reduced the oxygen concentration throughout the liquid down to this limiting value. The aggregations will then form because the flagellates have become positively chemotropic to the lower concentration of oxygen at the center of the liquid.

Once established, such an aggregation of flagellates does not remain long in the same form. An area free from flagellates appears at the center of the aggregation so that the organisms lie in a circular band surrounding the clear area. The latter increases in size and its bordering band of flagellates in diameter, the band gradually becoming less circular and more square in shape, if the cover-glass is a square one. The clear central area is a region where the oxygen consumption of the flagellates has reduced the oxygen content to such a low value that the organisms are forced to leave the region. They collect in a band where the concentration of dissolved oxygen is an optimum for them. It is the equilibrium position between the oxygen consumed at the center and that diffusing in from the edges of the liquid. As the consumption at the center is more rapid than the replacement from the edge, the flagellate band moves outwards until it becomes stationary at a position where the rates of consumption and replacement of oxygen are equal.

Although the flagellates collect in this manner in regions of optimum oxygen concentration, yet greater concentrations of dissolved oxygen have no injurious effect on them. Concentrations of dissolved oxygen lower than the optimum have the effect of inhibiting the movement of the flagellates. They recover their activity, however, immediately they are given access to dissolved oxygen again.

Work done in the past on chemotropism of flagellates will have to be revised in the light of the above facts, since the oxygen content of solutions used has never been taken into account.

The work was done during the months of June to September, 1919, at the Laboratory of the Marine Biological Association, Plymouth. I wish to thank the Director and Staff for the help and advice given to me. I wish also to thank the Trustees of the Ray Lankester Fund for having nominated me to an Investigatorship. Mr. C. Clifford Dobell F.R.S. was kind enough to identify the species and examine fixed preparations for me.

THE EQUILIBRIUM BETWEEN HEMOLYTIC SENSITIZER AND RED BLOOD CELLS IN RELATION TO THE HYDROGEN ION CONCENTRATION.

By CALVIN B. COULTER.*

(From the Hoagland Laboratory, Brooklyn.)

(Received for publication, December 22, 1920.)

INTRODUCTION.

The reversible nature of the union between various kinds of antigen and antibody in the usual physiological saline solution has been demonstrated many times.[1–12] Landsteiner and Jagić[3] have interpreted the reaction between antigen and antibody as a reversible reaction which is essentially similar to that taking place between simpler and definitely known chemical substances and in which an equilibrium is reached depending upon the concentration of the reacting substances and the temperature. Further evidence that suggests such an equilibrium state is given by Bail[9] and Matsui[11] in experiments on the splitting off of anticholera sensitizer. However, this point cannot be regarded as definitely proved by experiment.

That the chemical reaction, or the acidity or alkalinity, is a factor in the combination of antibody and antigen appears in any event

* Van Cott Fellow in Pathology.

[1] Landsteiner, K., *Münch. med. Woch.*, 1902, xlix, 1905.

[2] Morgenroth, J., *Münch. med. Woch.*, 1903, l, 61.

[3] Landsteiner, K., and Jagić, N., *Münch. med. Woch.*, 1903, l, 764.

[4] Landsteiner, K., and Jagić, N., *Münch. med. Woch.*, 1904, li, 1185.

[5] Landsteiner, K., and Reich, M., *Centr. Bakt., Ite Abt., Orig.*, 1905, xxxix, 83, 712.

[6] Bail, O., and Tsuda, K., *Z. Immunitätsforsch., Orig.*, 1908–09, i, 546.

[7] Tsuda, K., *Z. Immunitätsforsch., Orig.*, 1909, ii, 225.

[8] Spät, W., *Z. Immunitätsforsch., Orig.*, 1910, vii, 712.

[9] Bail, O., *Z. Immunitätsforsch., Orig.*, 1914, xxi, 202.

[10] Bail, O., and Rotky, K., *Z. Immunitätsforsch., Orig.*, 1913, xvii, 566.

[11] Matsui, I., *Z. Immunitätsforsch., Orig.*, 1915, xxiii, 233.

[12] von Liebermann, L., *Biochem Z.*, 1907, iv, 25; *Arch. Hyg.*, 1907, lxii, 277.

from a number of investigations. Von Liebermann[12] found that small amounts of alkali inhibit the action of hemolytic sera, while acid in small amount increases this action and in larger amount inhibits it. Hecker,[13] Sachs and Altmann,[14] and von Eisler[15] have confirmed this effect of alkali. Rondoni[16] has carried the analysis of this phenomenon farther and has shown the action of alkali in inhibiting the union of hemolytic sensitizer and red cells, and a similar but less marked effect of acid, which did not depend apparently upon destruction of the immune substance but rather upon a reversible modification of it.

The influence of reaction has been shown likewise on the dissociation or splitting off of antibody from combination with its antigen. Hahn and Trommsdorf[17] were able to separate agglutinin from sensitized bacteria by digestion with N/100 NaOH and almost as well with N/100 H_2SO_4, while physiological saline solution was found ineffective. Von Liebermann and von Fenyvessy[18] effected the separation of hemolytic sensitizer from sensitized cells by dilute H_2SO_4. Rondoni[16] found that digestion of sensitized cells with alkali or acid yielded a larger amount of free sensitizer than did a like volume of physiological saline solution. The separation in alkali was more complete than in acid; in several experiments with alkali approximately 60 per cent of the total sensitizer in combination was obtained free.

The presence of electrolytes appears also to be concerned in this combination. Although Ferrata[19] and Sachs and Teruuchi[20] have shown that sensitizer and cells will combine in a salt-free medium, von Eisler[21] found that the combination is less rapid and complete in salt-free than in salt-containing media. Kosakai[22] has found

[13] Hecker, R., *Arb. Inst. Exp. Therap. Frankfort*, 1907, iii, 39.

[14] Sachs, H., and Altmann, K., *Berl. klin. Woch.*, 1908, xlv, 699.

[15] von Eisler, M., *Centr. Bakt., Ite Abt., Orig.*, 1908, xlvi, 353.

[16] Rondoni, P., *Z. Immunitätsforsch., Orig.*, 1910, vii, 515.

[17] Hahn, M., and Trommsdorf, R., *Münch. med. Woch.*, 1900, xlvii, 413.

[18] von Liebermann, L., and von Fenyvessy, B., *Centr. Bakt., Ite Abt., Orig.*, 1908, xlvii, 274.

[19] Ferrata, A., *Berl. klin. Woch.*, 1907, xliv, 366.

[20] Sachs, H., and Teruuchi, Y., *Berl. klin. Woch.*, 1907, xliv, 467, 520, 602.

[21] von Eisler, M., *Z. Immunitätsforsch., Orig.*, 1909, ii, 159.

[22] Kosakai, M., *J. Immunol.*, 1918, iii, 109.

extraction with isotonic saccharose solution an effective means for separating sensitizer from cells.

Michaelis and Davidsohn[23] have endeavored to relate the phenomenon of specific agglutination to the electrical properties of the reacting substances, but state that typhoid bacilli and immune serum combine readily when the particles of both are negatively charged, so that their union cannot depend upon an affinity due to opposite electrical charge. They conclude further that specific typhoid agglutination and precipitation are independent of the hydrogen ion concentration—a conclusion which if applied to the general reaction between antigen and antibody would seem at variance with the facts related above.

We have shown in an earlier paper[24] that the agglutination of sensitized sheep cells has an optimum at pH 5.3 at which point the occurrence of agglutination is independent of the presence of electrolyte. This point does not coincide with the isoelectric point of the cells which was found for both the normal and sensitized cells to be about pH 4.65, and the suggestion was made that the optimum for agglutination is related to the isoelectric point of the immune serum. The present work is an outcome of the earlier investigation and is concerned with the relation of the hydrogen ion concentration to the union of hemolytic sensitizer and cells.

EXPERIMENTAL.

The methods employed were similar to those already described. To investigate the combination of sensitizer with cells in the absence, as far as possible, of electrolyte, sheep cells were washed in four changes of isotonic saccharose solution after washing in saline solution. The cells were made up to 10 per cent by volume of the concentrated sediment in saccharose solution. A series of eight to ten tubes was prepared, each containing 5 cc. of isotonic saccharose solution (9.2 per cent) and varying amounts by drop addition of N/10 NaOH or N/10 HCl. To each tube were then added precisely 0.1 cc. of undiluted immune rabbit serum and immediately afterward

[23] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xlvii, 59.

[24] Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 309.

5 cc. of 10 per cent cell suspension. The tubes were stoppered with paraffined corks, gently agitated, and kept in the water bath at 38°C. for 35 minutes, with gentle agitation every 5 minutes. The tubes were then centrifugated and the supernatant fluid was drawn off into two equal portions. To the first portion indicator was added and the pH determined colorimetrically by comparison with a standard series. The second portion served as a color screen for the standard tube. After the determination of the pH value, the first tube was titrated with N/100 HCl or N/100 NaOH to a pH between 6.5 and 6.0 and the amount of acid or alkali so required added to the second portion of test fluid. This portion was then diluted with saline solution and its content of sensitizer determined by titration in the usual way, with physiological saline solution as the medium. 0.04 cc. of guinea pig serum was used as complement. The cells used were 0.5 cc. of a 3 per cent suspension of the same sheep cells used in the first part of the experiment. The total volume of each tube was 2.0 cc. In consequence of the adjustment of the reaction of the test fluid and the buffer action of the complement added the hydrogen ion concentration of the tubes in this titration was sensibly constant. For each experiment the immune serum itself was titrated directly, using the same saline solution, complement, and cells. The hemolytic value was determined by interpolation, as, for instance, where with a 1:10 dilution of the supernatant test fluid the readings were 0.4 cc. complete, 0.35 cc. almost complete, 0.3 cc. ±; the value chosen was 0.375 cc. By this means and by the use of a 3 per cent cell suspension the error is within 5 per cent of the true value for any given reading. In the calculations the alteration in volume of the test fluid by the two additions of acid or alkali was taken into consideration.

The effect of electrolyte was determined by adding 1 or 2 cc. of physiological saline solution together with 4 or 3 cc. of saccharose solution to a series of tubes and then adding acid or alkali, sensitizer, and cells as before.

The dissociation of sensitizer from cells was investigated as follows: 5 cc. of concentrated cell sediment from saccharose were sensitized with approximately 50 units of sensitizer per unit of cells in a volume of 50 cc. Saccharose solution was used as the medium so that the

only electrolyte present was that added with the immune rabbit serum. The mixture of cells and serum was left standing for 30 minutes in the water bath at 38°C. and 1 hour in the refrigerator at about 8°C., then centrifugated, and the supernatant fluid drawn off. The pH of this was usually about 6.5. The cells were then washed once or twice in saccharose solution and made up to 10 per cent strength. From this point the procedure was the same as for the combination of sensitizer except that the addition of 0.1 cc. of sensitizer was omitted. The supernatant fluids from the sensitization and

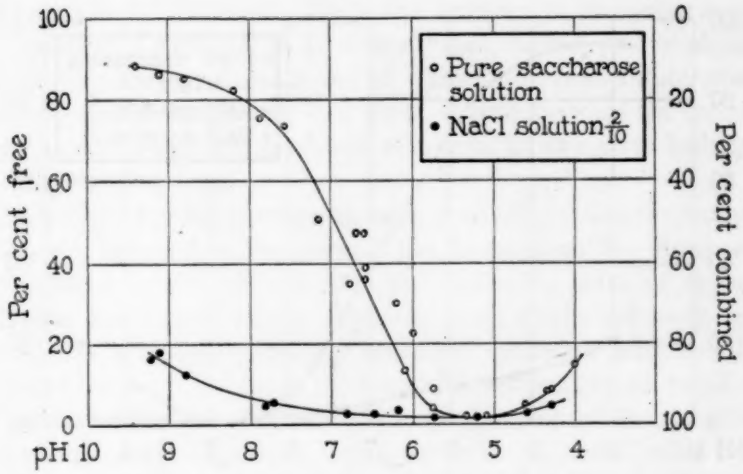

FIG. 1. Curve showing the proportion of the total sensitizer present either free or combined with cells, when the two combine *de novo*.

from the washing of the sensitized cells were titrated for their content of antibody and the sum of these values subtracted from the value of the immune serum itself. The remainder gives the amount of sensitizer actually in combination with the cells.

The results of both series of experiments are given in the form of curves, Fig. 1 for combination and Fig. 2 for dissociation. The abscissæ represent pH values, the ordinates the percentage of the total amount of sensitizer present either free or combined with cells. The curves show that near pH 5.3 the amount of sensitizer free in the supernatant fluid is at a minimum and the amount combined with the

cells is at a maximum. On the alkaline side of this point in the absence of electrolyte the percentage of sensitizer uncombined increases with the alkalinity and reaches a maximum of nearly 100 per cent at about pH 10. On the acid side of pH 5.3 the percentage of sensitizer uncombined increases with the acidity but somewhat less rapidly than for a corresponding increase in alkalinity.

It is impossible to carry the observations to reactions more acid than pH 4 on account of hemolysis. The fragility of heavily sensitized cells is well known; both normal and sensitized sheep cells

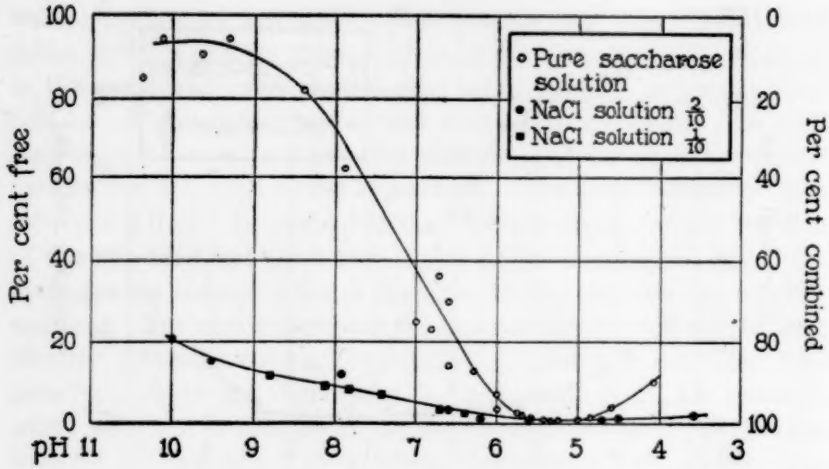

FIG. 2. Curve showing the proportion of the total sensitizer present either free or combined with cells, when the sensitizer dissociates from combination.

can endure, in the absence of electrolyte, a reaction of pH 4.5 without a trace of hemolysis provided they are not agitated. At this and more acid reactions a considerable degree of hemolysis can be caused by shaking or even gentle agitation. The agitation to which the cells were subjected while in the water bath caused a trace of hemolysis at all reactions; this hemolysis was apparently no greater at pH 10 than at pH 7.4, the normal reaction of the blood. Numerous observations were made on the acid side of pH 5 which are not recorded because of the difficulty in satisfactory colorimetric determination

of the pH value in consequence of hemolysis. All agree, however, in showing an increased dissociation with increased acidity.[25]

The presence of electrolyte as NaCl greatly increases the proportion of sensitizer combined with cells at all reactions except those near pH 5.3. At this point the combination of sensitizer with cells is independent of the presence of electrolyte. This recalls the observation, to which reference has already been made, that the agglutination of sensitized cells is independent of electrolyte at pH 5.3; it occurs as readily in the presence as in the absence of salt.

From a comparison of the two curves (Figs. 1 and 2) it is seen that they are almost identical. Since the volumes in the experiments are practically constant it is evident that, under the conditions both of combination *de novo* and of dissociation from combination, an equilibrium is established in a given volume between the amount of sensitizer free and that combined with cells, for any given hydrogen ion concentration.

The isoelectric point of serum globulin in which fraction the immune bodies are believed to be carried has been given by Rona and Michaelis[26] as about pH 5.4, and the isoelectric point of typhoid agglutinin has been found by Michaelis and Davidsohn[23] to lie near pH 5.2. It is probable that the hemolytic sensitizer used here has the same value. The point of maximal combination of sensitizer and cells coincides therefore with the isoelectric point of the sensitizer.

The amphoteric electrolytes, with which the immune bodies must be classed on the basis of their behavior in the electric field (Michaelis and Davidsohn;[23] Landsteiner and Pauli[27]), owe their electrical charge

[25] It was found that no destruction or irreversible modification of the sensitizer is brought about by the degrees of acidity or alkalinity reached in the experiments. A series of tubes, each containing 0.1 cc. of sensitizer in a volume of 10 cc. of saccharose, was brought to various reactions corresponding to those in the experiments with cells, and kept at 38°C. for 35 minutes. After centrifugation the supernatant fluids were adjusted in reaction and titrated for antibody content. No significant differences were found between any of the tubes; the differences were within the experimental error.

[26] Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193.

[27] Landsteiner, K., and Pauli, W., *25th Kong. Inn. Med.*, cited by Landsteiner, K., Kolloide und Lipoide in der Immunitätslehre, in Kolle, W., and Wassermann, A., Handbuch der Pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 1241.

to ionization. On the alkaline side of the isoelectric point they ionize as acids, on the acid side as bases; at the isoelectric point the ionization is at a minimum. It is evident that the combination of sensitizer and cells is related intimately to the ionization of the immune body. The curves showing the fraction of sensitizer free in solution in a salt-free medium follow closely the curves given by Sörenson,[28] after Michaelis,[29] to represent the degree of ionization of an amphoteric electrolyte. The ionized fraction of the sensitizer, both as anion and as cation, corresponds with that fraction which is uncombined with cells, so that we may conclude that the cells combine only with the undissociated molecules of sensitizer.

The ionization of the cells appears not to be a factor in their combination with sensitizer. There is no inflection in the curves at pH 4.6, the isoelectric point of the cells. At pH 5.3, the reaction at which the maximal amount of sensitizer is combined, the cells are considerably ionized as is demonstrated in the curve showing their rate of movement in the electric field.[24]

On the alkaline side of the isoelectric point proteins combine only with cations.[30] In the presence of NaCl, a Na salt could be formed therefore with the immune body at reactions more alkaline than pH 5.3. If this salt had a small dissociation constant, so that only a small concentration of ampholyte anion could exist in the presence of Na without combining to form undissociated salt, the degree of ionization of the ampholyte would be represented by such a curve (Michaelis[31]) as that showing the proportion of sensitizer uncombined in the presence of NaCl. While we possess no information as to the degree of dissociation of such a Na sensitizer salt, the effect of NaCl is at least suggestive of a depression in the ionization of the sensitizer with combination between the cells and all undissociated molecules of sensitizer, either pure or united with cation to form a salt.

[28] Sörenson, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.
[29] Michaelis, L., *Biochem. Z.*, 1911, xxxiii, 182.
[30] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559.
[31] Michalis, L., *Biochem. Z.*, 1920, ciii, 225.

CONCLUSIONS.

1. In a salt-free medium the proportion of the total amount of hemolytic sensitizer present, combined with the homologous cells, reaches a maximum of almost 100 per cent at pH 5.3. On the alkaline side of this point the proportion combined diminishes with the alkalinity and reaches a minimum of approximately 5 per cent at pH 10. On the acid side of pH 5.3 the proportion combined diminishes with the acidity but somewhat less rapidly than for a corresponding increase in alkalinity.

2. The presence of NaCl greatly increases the proportion of sensitizer combined with cells at all reactions except those in the neighborhood of pH 5.3. At this point the combination of sensitizer with cells is independent of the presence of electrolyte.

3. The curves representing the proportion of sensitizer combined or free run almost exactly parallel, both when the sensitizer combines *de novo* and when it dissociates from combination; therefore, in constant volume, at a given hydrogen ion concentration, and at a given temperature, an equilibrium exists between the amount of sensitizer free and that combined with cells.

4. The combination of sensitizer and cells is related fundamentally to the isoelectric point of the sensitizer.

5. The dissociated ions of the sensitizer, formed either by its acid or its basic dissociation, do not unite with cells. Combination takes place only between the cells and the undissociated molecules of the sensitizer.

SUMMATION OF DISSIMILAR STIMULI APPLIED TO LEAFLETS OF SENSITIVE BRIER (SCHRANKIA).

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(Received for publication, January 15, 1921.)

The writer has made experiments upon the leaflets of *Schrankia uncinata* Willd, or sensitive brier (Fig. 1), to determine whether subminimal stimuli of different character, such as chemical and mechanical, when applied simultaneously would combine their effects so as to reinforce each other and produce a visible response. *Schrankia* appeared suitable for this purpose because the closure of one leaflet mechanically stimulates the next distal leaflet and causes it to close also (this will be referred to as secondary stimulus), and thus a wave of closure is started which does not stop until all distal leaflets on the same side of the mid-rib are closed. Moreover, the intensity of the secondary stimuli is uniform as regards the leaflets of any given pinna, but varies according to the time of day. The intensity of the secondary stimulus is insufficient in the early morning to produce a visible response, but in the late afternoon it is sufficient to provoke prompt closure of the distal leaflets in turn. It was believed therefore that by choosing different times of the day for the experiment the intensity of the mechanical stimulus due to the effect of one leaflet on another could be varied at will, and that, at some optimum hour, the stimulating effect referred to would barely fall short of provoking a response. When this hour was found by actual trial in each individual case, it was planned to apply subminimal stimulation and then to observe whether the closure of one leaflet was followed by the closure of the next distal leaflet; that is, in the early morning, a single leaflet of *Schrankia* can be caused to close against the next distal leaflet without causing the latter to close (Fig. 2). In the late afternoon, on favorable days, the closure of any proximal leaflet inevitably provokes closure of the next distal leaflet (Fig. 3). The problem was

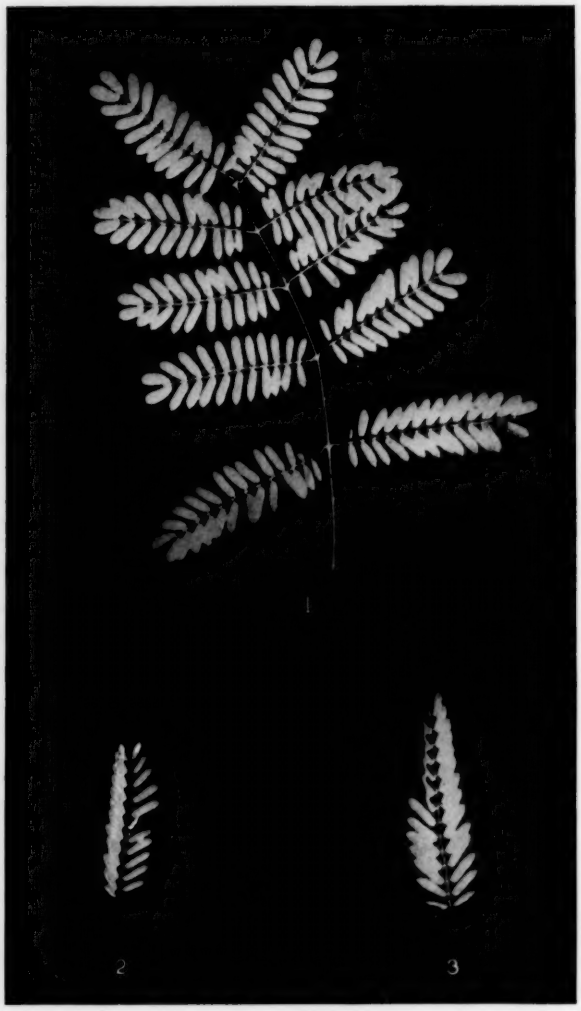

FIG. 1. Silhouette of leaf fastened (by a quick movement) between panes of glass and then fixed by heat to prevent closure of leaflets. Five pairs of pinnæ are shown, each consisting of about a dozen pairs of leaflets.

FIG. 2. Silhouette made artificially to show appearance of pinna after a single leaflet has been caused to close by stimulation. This result usually is seen in the forenoon.

FIG. 3. Silhouette made under artificial conditions to show appearance of pinna after wave of closure has involved all leaflets distal to the leaflet originally stimulated. This result is usually seen in the late afternoon.

to ascertain whether subminimal chemical stimulation could be so applied as to lower the threshold of susceptibility to stimulation and cause the leaflets to behave in the forenoon as they normally behave in the afternoon.

Several attempts were made to transplant *Schrankia*, but such treatment interfered with its reactivity. A spray of the plant was isolated within an Erlenmeyer flask, with the object of securing a definite concentration of chemical substance (chlorine gas or ammonia fumes) in the atmosphere surrounding the leaflets; but this experiment proved a failure because of condensation of water on the inner surface of the flask. Attempts were made to summate subminimal mechanical stimuli with subminimal stimulation due to the concentration by a lens of the rays of the sun, and to summate mechanical stimuli with stimulation of different acids, but these experiments failed.

Finally, on June 22, 1919, at 11.15 a.m., the following experiment was performed. Several pinnæ of a plant growing in the open were tested by touching a more or less proximal leaflet, and in each instance, the touched leaflet closed without inducing closure of its next distal neighbor. A petri dish containing ammonia water (27 per cent) was then placed carefully underneath the adjacent compound leaf (consisting of five pairs of pinnæ) and after a few seconds, a single leaflet of each of four pinnæ was touched. In every instance, all leaflets, on the same side of the mid-rib, lying distal to the touched leaflet closed in a regular succession or wave. These results were confirmed by repeating the experiment on June 24, at 4 p.m.

On July 3, in the forenoon, several pinnæ of an individual plant were tested by touching one leaflet of each pinna. In every instance, the touched leaflet closed without causing closure of the next leaflet. A stream of chlorine gas mixed with air (10 per cent chlorine) was then allowed to flow gently over each of several pinnæ taken in turn. 6 seconds after the beginning of the application of the chlorine mixture, a proximal leaflet was touched; it closed and caused the closure of the next distal leaflet. The wave of closure proceeded until all distal leaflets on the same side of the mid-rib were closed. The fact that the leaflets on the opposite side of the mid-rib did not close was accepted as proof that the chlorine mixture did not alone cause the closure of the distal leaflets. All of several leaflets tested gave similar results.

SUMMARY AND CONCLUSIONS.

1. The pinnæ of sensitive brier or *Schrankia* are favorable for demonstrating summation of dissimilar stimuli.

2. The demonstration was made as follows: A time of day was chosen when the closure of a single proximal leaflet did not provoke closure of the next distal leaflet. An irritating gas was applied to the pinna. A few seconds later, a single leaflet was touched; it closed, induced closure of the next distal leaflet, and inaugurated a wave of closure which proceeded until all leaflets on the same side of the mid-rib were closed.

COMPARATIVE STUDIES ON RESPIRATION.

XV. The Effect of Bile Salts and of Saponin upon Respiration.

By MATILDA MOLDENHAUER BROOKS.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, January 15, 1921.)

It was pointed out by Osterhout[1] that substances which increase the electrical conductivity of tissues are antagonized by those which decrease it. An illustration of this is seen in antagonism between NaCl (which increases conductivity) and Na taurocholate[2] (which decreases it). This case has additional interest as an example of antagonism between anions as well as of antagonism between organic and inorganic substances.

The experiments on electrical conductivity indicated that Na taurocholate acts like $CaCl_2$ in decreasing conductivity and in antagonizing NaCl. Whether this similarity would be found if other criteria were used remained an open question.

The experiments of the writer[3] showed that, when respiration is employed as a criterion, $CaCl_2$ antagonized NaCl. Similar results were obtained by Gustafson.[4] In view of this it became especially interesting to study the behavior of tissues under the influence of Na taurocholate.

In the experiments which were made for this purpose the organism studied was *Bacillus subtilis* and the technique was similar to that described in previous papers.[5] The results are shown in the figures.

Fig. 1 shows the effects of sodium taurocholate (0.0000125, 0.000015, 0.001, 0.0025, and 0.01 M) upon the rate of respiration (expressed as per cent of the normal rate). This organic salt is similar in its

[1] Osterhout, W. J. V., *Science*, 1915, xli, 255.
[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 405.
[3] Brooks, M. M., *J. Gen. Physiol.*, 1919–20, ii, 5.
[4] Gustafson, F. G., *J. Gen. Physiol.*, 1919–20, ii, 17.
[5] Brooks, M. M., *J. Gen. Physiol.*, 1919–20, ii, 5, 331.

toxicity to lanthanum nitrate in that very dilute solutions produce pronounced effects. There is slight increase in the rate in 0.0000125 M; the respiration is normal in 0.000015 M, while in higher concentrations there is a decrease in the rate. In calculating the molecular

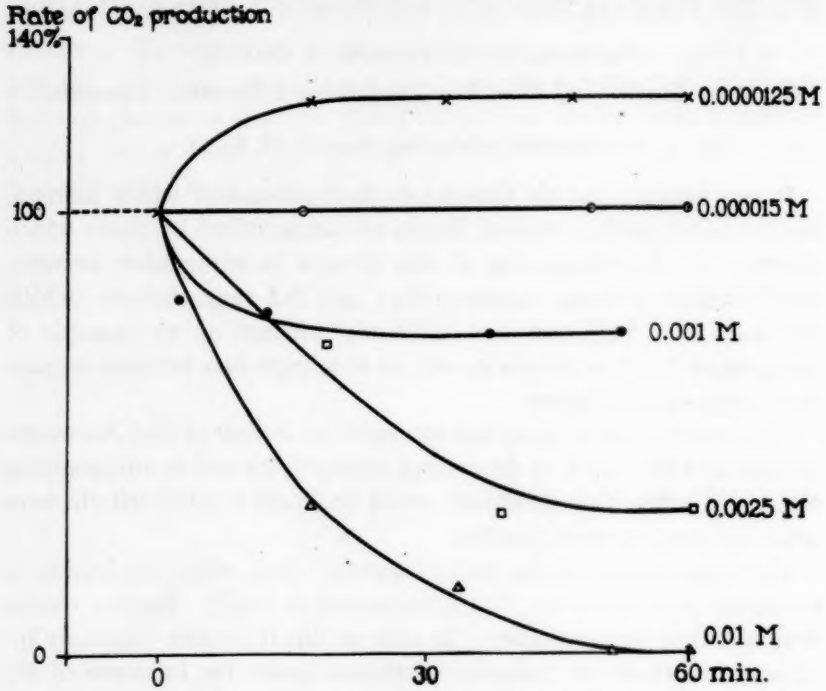

FIG. 1. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.0000125, 0.000015, 0.001, 0.0025, and 0.01 M Na taurocholate. The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Each curve represents a single typical experiment.

concentrations of this salt an approximate estimate only could be made because the purity of the salt is doubtful. In making up the solutions 1 gm. of sodium taurocholate in 100 cc. of distilled water was calculated to make about 0.02 M. During the first 10 minutes the

bacteria are under normal conditions and the curve (broken line) is horizontal. After this (at the point marked 0 on the abscissa) the salt is added. For example, the addition of sufficient Na taurocholate to make the concentration 0.0000125 M produces a rise in the rate, which remains constant during the period of experimentation. These curves are selected from a number of similar typical curves, and each represents one experiment.

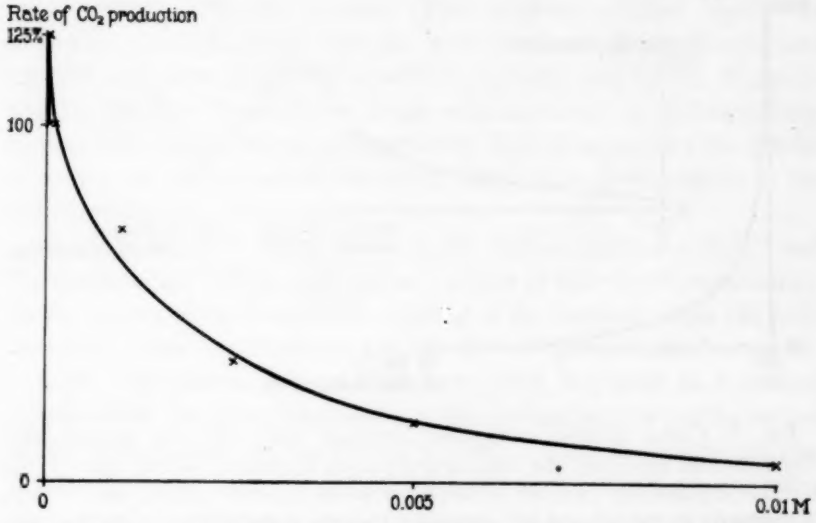

FIG. 2. Curve showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) as affected by Na taurocholate. Normal rate as in Fig. 1. Average of three experiments; probable error of the mean less than 2 per cent of the mean.

Fig. 2 shows the effects of various concentrations of sodium taurocholate upon the rate of respiration, expressed as per cent of the normal rate. The rate indicated is that produced after the bacteria had been in contact with the salt for 1 hour. The figure shows that sodium taurocholate produces an increase in the rate at a concentration of 0.0000125 M and a decrease in concentrations higher than 0.000125 M. It is of interest to note that the concentrations which are most favorable to the respiration of *Bacillus subtilis* are very dilute. The abscissæ represent the concentrations of the salts used, the ordinates represent the rate of respiration.

Fig. 3 shows antagonism between NaCl and Na taurocholate. In this figure the abscissæ represent the molecular proportions of each component in the mixture: thus the ordinate at the extreme left represents the rate in a pure solution of NaCl; the ordinate at the extreme right, the rate in a solution containing 95 parts of NaCl and 5 parts of Na taurocholate. In a solution containing 1 part of Na taurocholate

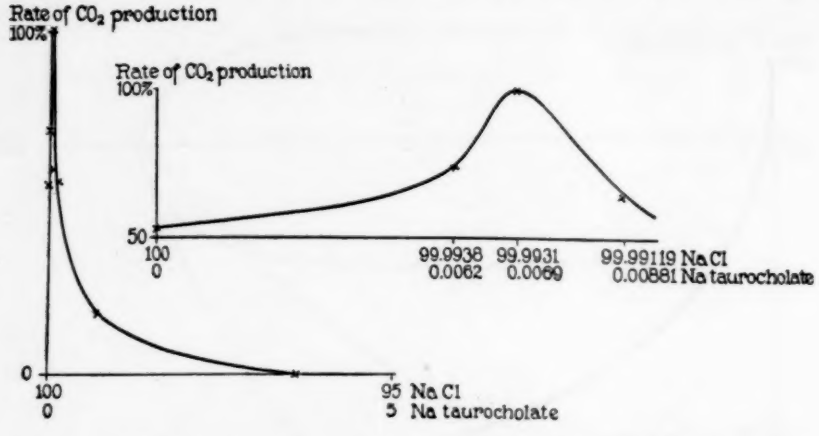

FIG. 3. Curve showing antagonism between NaCl and Na taurocholate. The ordinates represent the rate of respiration (expressed as per cent of the normal); the abscissæ represent molecular proportions of the solution used. Thus the ordinate at the extreme left represents the rate in 0.8 M NaCl while the ordinate at the extreme right (in the lower figure) represents the rate in 95 parts of NaCl and 5 parts of Na taurocholate (molecular proportions). Mixtures in which the proportion of Na taurocholate was greater showed no respiration.

Since the changes near the beginning of the curve could not be shown accurately this portion is represented on an enlarged scale in the upper part of the figure. The optimum proportion for the production of CO_2 is 99.9931 parts of NaCl and 0.0069 parts of Na taurocholate. Normal rate as in Fig. 1. Average of three experiments; probable error of the mean less than 2 per cent of the mean.

to 14,375 parts of NaCl the rate of respiration was practically normal. In all other proportions it was less than normal. For the sake of clearness, the portion of the figure at the extreme left has been drawn on an enlarged scale and is inserted above. These proportions are quite different from those found by Osterhout (500 of NaCl to 1 of Na taurocholate) to be most favorable, but the difference may be due to the

fact that in the experiments reported by Osterhout the highest proportion of NaCl used was 500 to 1. Since this shows good antagonism, higher proportions were not tried, as the object of the experiment was merely to demonstrate antagonism and not to ascertain the most favorable mixture.

Owing to the unusual results obtained with high dilutions of Na taurocholate, it was thought advisable to procure Na taurocholate from several different sources. The original product used was Merck's. An additional sample was obtained from Eimer and Amend, and some purified bile salts were kindly sent by Dr. Morgulis and Dr. Hecht. Experiments made with all the above different samples were in remarkably good agreement, thus showing that the results were not due to the impurities which might have been present in the Na taurocholate.

Experiments on recovery were made with a mixture of NaCl and Na taurocholate (which produced a decrease in the rate of respiration). In the course of an hour, after removal of the bacteria from the mixture to 0.75 per cent dextrose, the rate did not quite attain the normal.

A few experiments with saponin were tried, inasmuch as it seemed possible that the same mechanism might be responsible for the action of Na taurocholate and for that of saponin. Eighteen concentrations of saponin were used (ranging from 0.001 to 0.0000005 M), including those which produce an increase in the rate of respiration in the case of Na taurocholate. It was found that the concentrations which were greater than 0.00005 M produced a progressive decrease in the rate, while those which were less than 0.00005 M gave the normal rate of respiration (100 per cent). None gave an increase in the rate of respiration.

Both saponin and Na taurocholate are exceedingly effective in lowering surface tension but their effects on respiration are so different as to indicate that surface tension does not play an important part in this connection. This conclusion would not, however, be justified if it could be shown that saponin is unable to penetrate the cell. The fact that in the highest concentrations employed[6] there was some

[6] Higher concentrations were not employed on account of the foaming which they produced.

depression[7] of the rate of respiration indicates that saponin penetrates to some extent.

It is interesting to observe that the results obtained with *Bacillus subtilis* resemble those obtained by Osterhout in measuring the electrical conductivity of *Laminaria*, since in both cases the effect of saponin[8] is very much less than that of Na taurocholate.

SUMMARY.

1. The addition of Na taurocholate produces an increase in the rate of respiration at a concentration of 0.0000125 M, and a decrease at 0.001 M and in higher concentrations.

2. NaCl is antagonized by Na taurocholate, the most favorable proportion being 14,375 parts of NaCl to 1 part of Na taurocholate (molecular proportions).

3. Solutions of saponin, at concentrations from 0.00005 M to 0.001 M, decrease the rate of respiration: lower concentrations produce no effect.

[7] The depression was not due to the buffer action of the saponin. This was shown by comparing the change in pH produced in the saponin solution and in distilled water when a solution of carbonic acid was added to each.

[8] Unpublished results of experiments on *Laminaria*.

COMPARATIVE STUDIES ON RESPIRATION.

XVI. Effects of Hypotonic and Hypertonic Solutions upon Respiration.

By O. L. INMAN.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

(Received for publication, January 15, 1921.)

Studies showing the effects of the osmotic pressure of the medium upon the organism have been numerous and various. Little work, however, has been done upon the relation of the production of carbon dioxide as affected by varying the osmotic pressure of the medium in which the organism lives.

Smith[1] reports that stems of *Tropæolum*, stems and roots of bean seedlings, and leaves of snowdrops, after deprivation of one-third to one-half of their total water by drying, respired more than normal plants. No increase in the respiration was observed on partly drying young stems of peony and asparagus.

Palladin and Sheloumova[2] observed that potato tubers, when allowed to lose water by drying in the air or by immersion in a 10 per cent solution of sodium chloride, in general showed a decrease in the amount of carbon dioxide produced.

Bailey and Gurjar[3] have investigated the respiration of seeds as related to the amount of water present. In general it is shown the respiration falls off as the moisture diminishes.

Warburg[4] stated that hypertonic solutions may increase the consumption of oxygen by fertilized eggs of the sea urchin as much as three or four times. Loeb and Wasteneys[5] repeated these experi-

[1] Smith, A. M., *Rep. Brit. Assn. Advancement Sc.*, 1915–16, lxxxv, 725.

[2] Palladin, V. I., and Sheloumova, A. M., *Bull. Acad. Sc. Petrograd*, 1918, 801, abstracted in *Chem. Abstr.*, 1918, xii, 1889.

[3] Bailey, C. H., and Gurjar, A. M., *J. Agric. Research*, 1918, xii, 685.

[4] Warburg, O., *Z. physiol. Chem.*, 1909, lx, 443.

[5] Loeb, J., and Wasteneys, H., *J. Biol. Chem.*, 1913, xiv, 469.

ments using a different species (*Strongylocentrotus purpuratus*) and found no increase when sufficient NaCl was added to increase the specific gravity of the sea water by about 50 per cent. They found an increase in the case of unfertilized eggs (as did Warburg) but they attributed this to the fact that the hypertonic solution causes the formation of a membrane. Unfertilized eggs which had already formed membranes (as the result of treatment with butyric acid) showed no increase in hypertonic solutions. Measurements by the writer of the specific gravity of the solution described by Loeb and Wasteneys showed it to be about 1.036, while that of the most concentrated solution used by the writer was much above this.

Using the method of measuring respiration described by Osterhout,[6] the writer undertook to measure the production of carbon dioxide while the osmotic pressure of the medium was changed sufficiently to be highly hypertonic on the one hand and quite hypotonic on the other. The marine alga *Laminaria agardhii* was used as the basis of most of the work. The results with *Ulva lactuca* were quite similar to those with *Laminaria*.

It was found that the carbonates of sea water interfered with the measurement of the carbon dioxide produced, and to overcome this difficulty the chamber containing the *Laminaria* was lined with filter paper dipped in artificial sea water (van't Hoff's solution, containing no carbonates). No other liquid was added but the chamber was so small that a very slight amount of evaporation sufficed to saturate the air. The change thereby produced in the concentration of the solution in the thallus was negligible and the cells may be regarded as bathed in a solution of constant concentration during the experiment. Uniform normal respiration was also obtained when the *Laminaria* was immersed in artificial sea water or placed in the respiration chamber without the addition of liquid or moistened filter paper.

The pieces of *Laminaria* were selected from fresh material as nearly uniform in size as possible and placed in the container. The normal rate of respiration was then obtained for each piece of tissue before it was treated. The pieces were then removed and exposed to the hypertonic or hypotonic solution for 5 minutes, at the end of

[6] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17.

which time they were placed in the chamber (with a little of the same liquid as that with which they had been treated) and the rate of respiration was then measured at intervals of 10 to 20 minutes, until the expiration of 60 minutes.

The concentrated solutions of sea water were obtained by slow evaporation of normal sea water both with and without the use of heat.

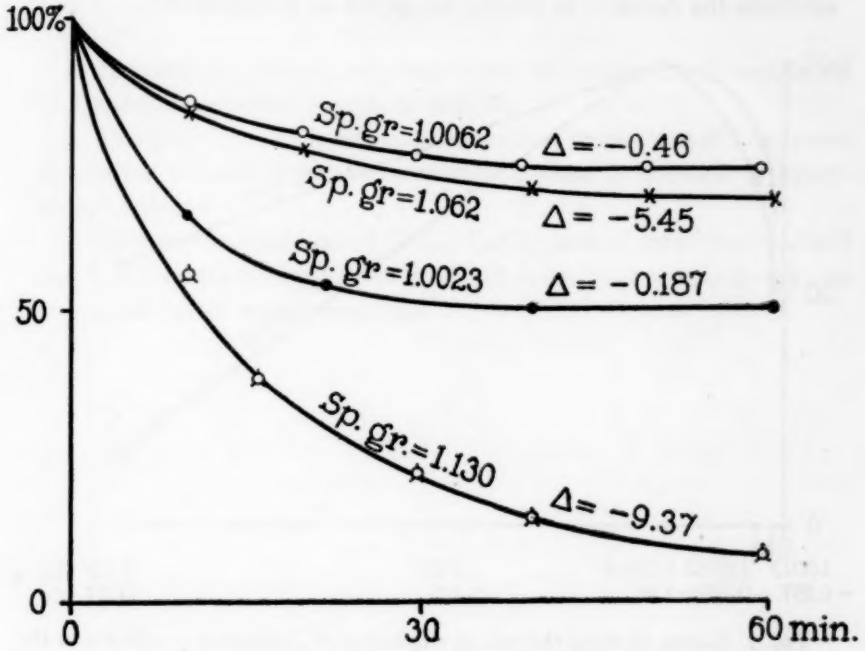

FIG. 1. Curves showing the rate of respiration of *Laminaria* during exposure to sea water of various specific gravities and freezing point depressions. Δ for Woods Hole sea water is normally − 1.81 and the specific gravity 1.024. The ordinates represent the rate of production of CO_2 expressed as per cent of the normal. The normal rate represents a change in pH from 7.78 to 7.36 in from 1.5 to 2 minutes. The abscissæ represent time in minutes. Average of 3 or more experiments: probable error of the mean less than 10 per cent of the mean.

No difference was found in the results they produced when of the same concentration. Artificial sea water (van't Hoff's solution, without the carbonates) in high concentration affected respiration in the same manner as ordinary concentrated sea water. The specific gravity of the sea water was measured by means of a Westphal balance and

the depression of the freezing point was approximately determined by means of a Beckmann apparatus. The temperature of these experiments was 18° ± 2°C.

Fig. 1 shows quite clearly that in higher concentrations, *e.g.* specific gravity 1.130 ($\Delta = -9.37$) there was always a decrease in the amount of carbon dioxide given off. It is also shown that with hypotonic solutions the decrease in respiration is not so pronounced.

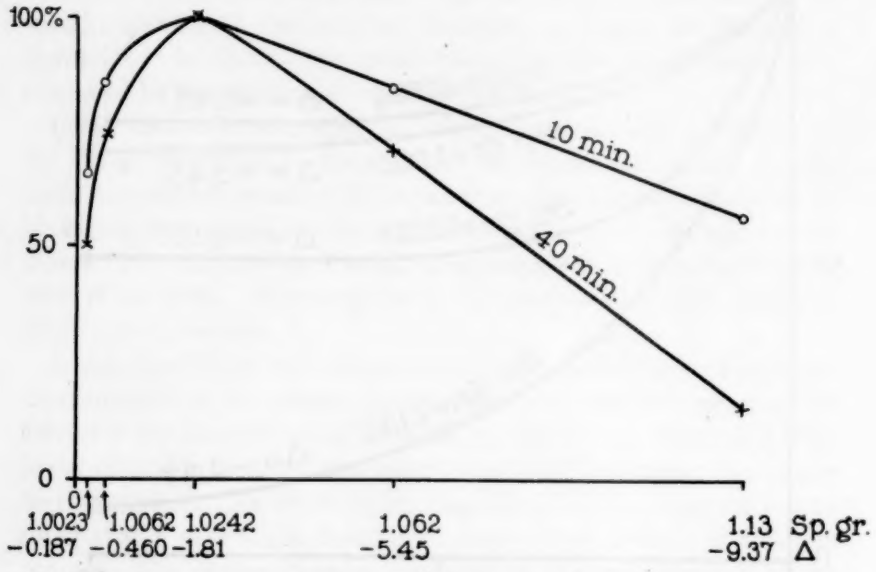

FIG. 2. Curves showing the rate of respiration of *Laminaria* in relation to the specific gravity and freezing point depression of sea water. The upper curve shows per cent of respiration at end of 10 minutes for all concentrations and dilutions. The lower curve shows the same at end of 40 minutes. The point at the intersection of the curves represents normal sea water. These curves are constructed from the same data as the curves in Fig. 1. The ordinates represent rate of production of CO_2 expressed as per cent of the normal. Abscissæ represent specific gravity; under each of the numbers denoting specific gravity is noted the corresponding freezing point depression.

Fig. 2 shows the relation between the specific gravity of the solution and the respiration to be almost a linear function in the case of hypertonic solutions. In the case of hypotonic solutions it is not so simple.

Experiments of a similar nature were carried out on wheat seedlings in which hypertonic solutions alone were used. Hypertonic solutions,

of the same specific gravity as sea water (specific gravity 1.024), of sodium chloride and calcium chloride were tried; also a mixture of sodium chloride and calcium chloride in the molecular proportion of 50:1. The results were all similar to those produced by the use of hypertonic sea water on *Laminaria* in that there was always a fall in the rate of respiration.

SUMMARY.

1. In highly hypertonic solutions of sea water the rate of respiration of *Laminaria agardhii* is rapidly reduced.

2. In highly hypotonic solutions the rate of respiration of *Laminaria agardhii* is reduced somewhat less rapidly than in the case of hypertonic solutions.

3. Hypertonic solutions of NaCl, $CaCl_2$, and of mixtures of NaCl and $CaCl_2$ in the proportion of 50:1, all caused a decrease in the rate of respiration of wheat seedlings.

FURTHER OBSERVATIONS ON THE PRODUCTION OF PARTHENOGENETIC FROGS.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 19, 1921.)

The writer has repeatedly published[1] short reports on the production of frogs from unfertilized eggs by Bataillon's method of puncturing the membrane of the egg with a fine needle. The writer has succeeded in raising over twenty of these parthenogenetic frogs to an advanced and some to an adult stage. Two such specimens (leopard frogs) are represented in Fig. 1, together with a scale giving their size. They were at the time of death 13 and 14 months old respectively, and the death of these, as of the other specimens, was due to intestinal infection. The parthenogenetic frogs were apparently normal in every respect.

The second point of interest is the fact that both sexes occur among the parthenogenetic frogs. Three females were obtained among over twenty males, yet the preponderance of males may have been simply an accident. Fig. 2 gives a macroscopic photograph of ovaries and kidneys of one parthenogenetic female, and Fig. 3 a microphotograph of a section through the ovary. The fact that both sexes occur suggests that in the frog the female may be heterozygous for sex.

It was ascertained that the male parthenogenetic frogs and tadpoles possess a diploid and not a haploid number of chromosomes. The writer had the good fortune of obtaining the expert advice of Professor Richard Goldschmidt,[2] and later of Doctor Parmenter,[3] on this problem. Both authors found unquestionably a diploid number of chromosomes in the males. Parmenter was able to count definitely twenty-

[1] Loeb, J., and Bancroft, F. W., *J. Exp. Zool.*, 1913, xiv, 275; 1913, xv, 379. Loeb, J., *Proc. Nat. Acad. Sc.*, 1916, ii, 313; 1918, iv, 60; The organism as a whole, from a physicochemical viewpoint, New York, 1916.

[2] Goldschmidt, R., *Arch. Zellforsch.*, 1920, xv, 283.

[3] Parmenter, C. L., *J. Gen. Physiol.*, 1919–20, ii, 205

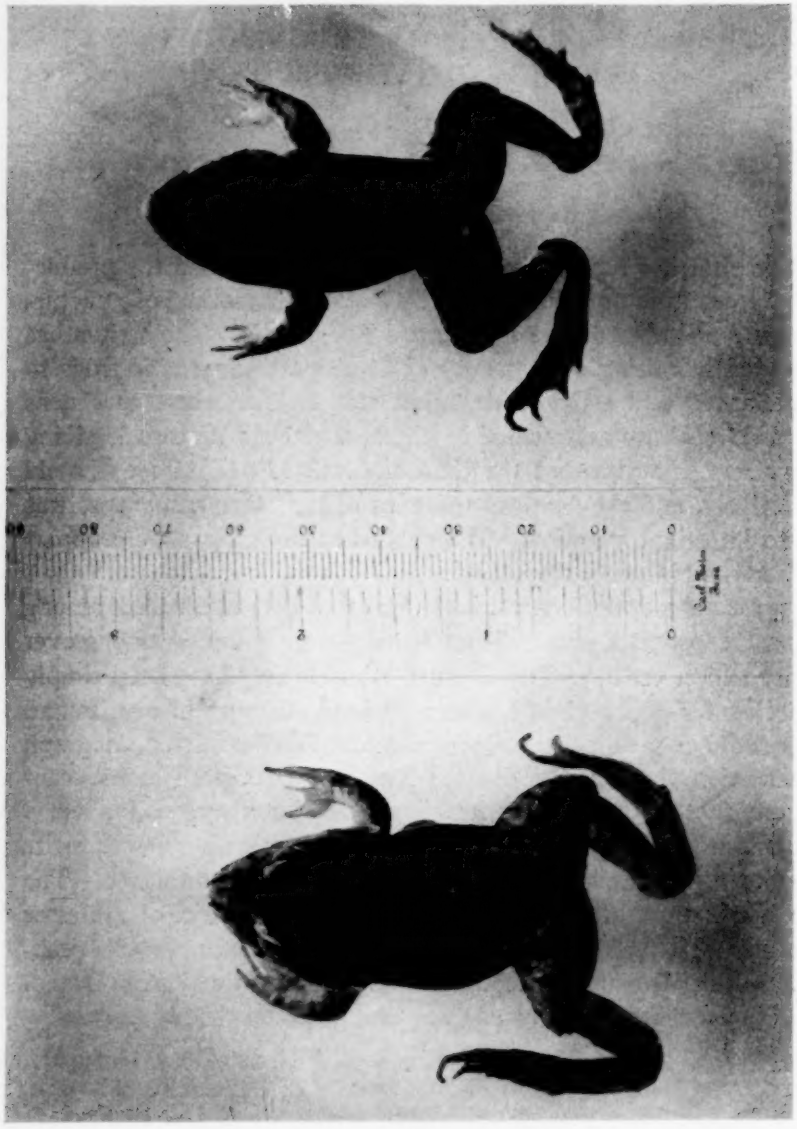

FIG. 1. Two adult male parthenogenetic leopard frogs, natural size (preserved in formaldehyde). The one to the left lived from Feb. 27, 1917, to Mar. 24, 1918; the one to the right from Mar. 16, 1916, till May 22, 1917

six chromosomes in a number of specimens. The question arises how to account for the fact that the number of chromosomes is diploid. The first thought might be that the eggs had been accidentally fertilized, but this is excluded by the mode of procedure. As is well known, the eggs of the frog are fertilized outside the body of the

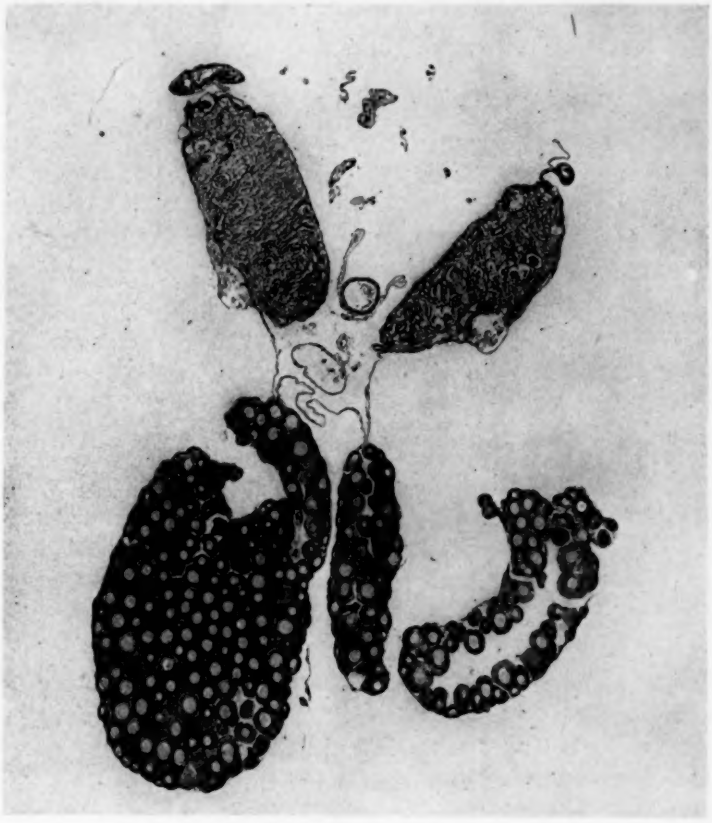

FIG. 2. Ovaries and kidneys of a parthenogenetic female frog.

female, and the females used for our experiments had not yet commenced to lay their eggs. The females after having been killed were submersed in 95 per cent alcohol and left there for several minutes to kill any sperm that might have stuck to the outside skin. The skin was cut open and the eggs were removed from the oviduct with

sterilized instruments. The hands of the experimenter were also sterilized. About 50 to 100 eggs were put on each glass slide and the eggs of every second or third slide were not punctured, serving as controls. In no case did a single control egg show any development;

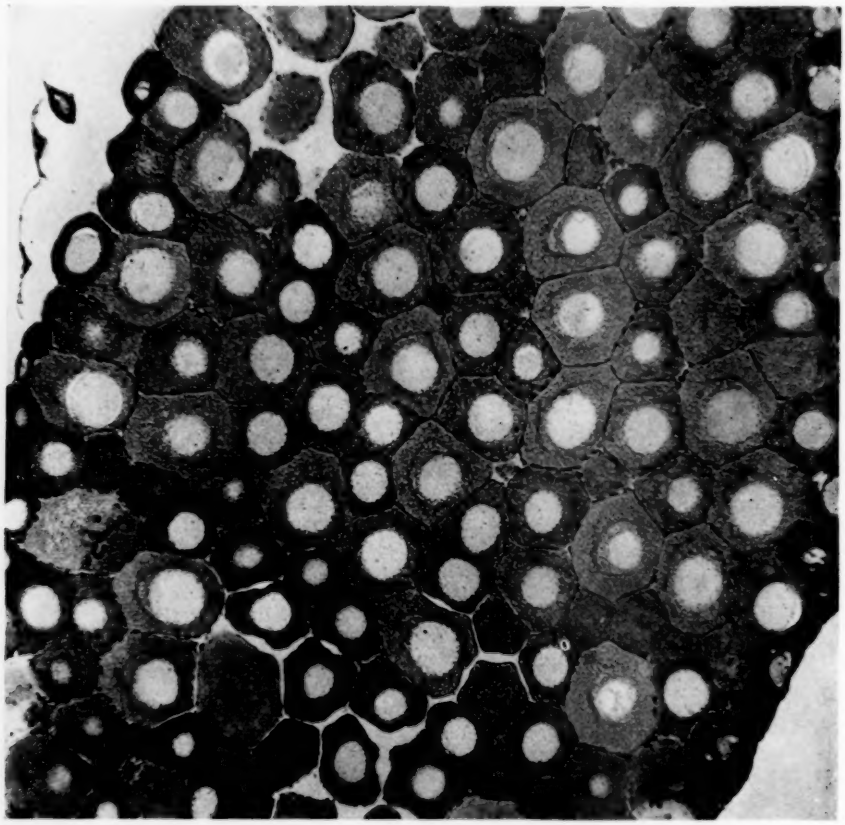

FIG. 3. Section through the ovary shown in Fig. 2.

only punctured eggs developed. This excludes the idea that the eggs were fertilized.

There remain other possibilities to account for the fact that the chromosome number is diploid. As Parmenter states[3]

"The diploid number, as well as the similarity in form of the tetrads of the parthenogenetic and normal animals, may have been brought about by the retention of the second polar body, or by a premature division of the chromosomes without the division of the cell body just before the first cleavage."

Brachet[4] had previously counted the number of chromosomes in a parthenogenetic tadpole, 18 days old, and found a diploid number, but, of course, it was out of the question to ascertain the sex of the tadpole.

In my last publication on the subject I mentioned the possibility that the parthenogenetic females might have the haploid number of chromosomes. This question can only be decided by an actual count of the chromosomes in female parthenogenetic frogs. It is perhaps of interest in this connection that Hovasse[5] in an investigation of the number of chromosomes of young parthenogenetic tadpoles of frogs reports to have found both the diploid number and the haploid number. Since it is not possible to determine the sex of early tadpoles the observations of Hovasse do not answer the question whether or not the female parthenogenetic frog possesses a haploid number of chromosomes. There is, however, no doubt left that some if not all of the parthenogenetic male frogs possess a diploid number of chromosomes.

We usually received in one shipment a large number of female frogs either from South Carolina or from Chicago as soon as the spawning season began. It took about half a day to puncture the eggs of one female frog and as a consequence some time elapsed before the eggs of every female in the lot were punctured. It was found that tadpoles of good vitality were obtained only from the eggs of the first and second frogs used for the experiment. The eggs of the frogs which were punctured later, after the frogs had been in the laboratory for 2 days or more, either gave no tadpoles at all or if tadpoles were produced they died in less than 3 weeks, while the tadpoles from the eggs punctured immediately after the arrival of the frogs went on developing although the conditions under which the eggs were kept were the same in all cases (see Table I).

[4] Brachet, A., *Arch. Biol.*, 1911, xxvi, 362.

[5] Hovasse, R., *Compt. rend. Acad. Sc.*, 1920, clxx, 1211.

TABLE I.

Date of experiment.	Female No.	Approximate No. of eggs punctured.	No. of tadpoles hatching.	No. of tadpoles that died within 3 weeks.
1919				
Mar. 25	I	8,500	92	21
" 26	II	5,000	43	22
" 28	III	3,000	9	9
" 29	IV	3,000	2	2
" 30	V	100	0	0
" 30	VI	400	0	0
" 30	VII	200	0	0
" 31	VIII	1,500	13	13
" 31	IX	150	0	0
" 31	X	1,300	1	1
" 31	XI	No eggs used.		
Apr. 1	XII	2,800	13	13
" 1	XIII	1,700	0	0
" 1	XIV	1,400	0	0
" 2	XV	1,200	16	14
" 2	XVI	200	0	0
" 2	XVII	1,100	1	1
" 3	XVIII	1,800	16	16
" 3	XIX	2,000	16	12
" 4	XX	1,500	0	0
" 4	XXI	None.		
" 4	XXII	1,700	0	0
" 5	XXIII	2,000	0	0

The results of a series of experiments made on the eggs of a second lot of frogs received later confirmed these results (Table II).

TABLE II.

Date of experiment.	Female No.	Approximate No. of eggs punctured.	No. of tadpoles hatched.	No. of tadpoles that died within 3 weeks.
1919				
Apr. 8	XXIV	2,000	6	5
" 8	XXV	4,000	14	14
" 9	XXVI	3,000	2	2
" 9	XXVII	3,500	0	0
" 10	XXVIII	2,000	0	0

It is, therefore, obvious that only those eggs were capable of developing which were punctured on the 1st or 2nd day while later they lost their power of developing or lost their vitality. It may be

possible that the eggs must be punctured at a certain stage, *e.g.* at the period after the first polar body is given off and before the second polar body is extruded in order to develop or in order to develop normally, but this can only be decided by further experiments.

In these experiments the eggs were not covered with blood since it was found in previous experiments that this did not improve the yield of parthenogenetic tadpoles.

Many, sometimes the majority, of the parthenogenetic tadpoles did not metamorphose even within a year although they grew normally. It was suspected that this was due to an imperfect development of the thyroid gland or to lack of iodine in the food. In one tadpole, over a year old, the attempt was made to bring about metamorphosis by the feeding of thyroid gland from cattle. Three feedings were sufficient to cause metamorphosis within 2 weeks.

CHEMICAL AND PHYSICAL BEHAVIOR OF CASEIN SOLUTIONS.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 6, 1921.)

1. L. L. Van Slyke and J. C. Baker described in 1918[1] a method for preparing "pure casein" from skimmed milk, which consisted in "the gradual addition of acid and its immediate distribution through the mass of milk without causing coagulation of casein at the point where the acid first comes into contact with a portion of the milk. This result can be accomplished by introducing the acid below the surface of the milk with simultaneous high-speed mechanical stirring. . . . After standing under gentle stirring for 3 hours with acidity just below the point of casein coagulation, addition of acid is continued slowly, accompanied as before by rapid stirring in order to obtain the particles of casein coagulum in the finest possible state of division." The coagulated casein is then centrifuged and after repeated washings is found free from Ca and P. As Van Slyke and Baker point out, the pH of this casein coagulum is about 4.5 to 4.6; *i.e.*, it is slightly below the isoelectric point. The essential feature of Van Slyke and Baker's method, therefore, consists in slowly bringing the milk or casein solution approximately to the pH of the isoelectric point of casein. The writer has shown that gelatin gives off all ionogenic impurities at the isoelectric point[2] and Van Slyke and Baker's experiments show that the same method works also with casein. The casein prepared after Van Slyke and Baker's method is also free from albumin since this latter protein is soluble at pH 4.5 or 4.7, and is hence removed from the insoluble isoelectric casein by washing.

[1] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 127.

[2] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

In our experiments we used casein prepared after Van Slyke and Baker's method from skimmed milk and in addition from a commercial "pure casein." Both preparations gave practically the same result. In order to remove traces of fat from the casein the latter was washed in acetone.

2. In previous publications the writer had shown that weak dibasic and tribasic acids combine in molecular proportions with crystalline egg albumin, prepared after Sörensen and with gelatin.[3] It can be shown that the same is true for casein. 1 gm. of isoelectric casein, prepared after Van Slyke and Baker, was put into 100 cc. of watery solution containing 1, 2, 3, etc. cc. of 0.1 N HCl or 0.1 N H_3PO_4. The pH of the casein solution was ascertained potentiometrically and the number of cc. of 0.1 N acid required to bring the 1 per cent casein solution to the same pH was plotted as ordinates over the final pH of the casein solution as abscissæ. The casein chloride or casein phosphate is not completely soluble in a 1 per cent solution at room temperature until the pH is about 3.0 or a trifle below. When too much acid is added, *i.e.* when the pH is 1.6 or possibly a little above, casein precipitates out again from a 1 per cent solution.

Fig. 1 gives the curves for HCl and H_3PO_4, drawn out within those limits of pH within which the casein salts are soluble in a 1 per cent solution at room temperature. The curves show that about three times as many cc. of 0.1 N H_3PO_4 as of 0.1 N HCl are required to bring 1 gm. of originally isoelectric casein in a 1 per cent solution to the same pH; or in other words, H_3PO_4 combines with casein in molecular proportions, as we should expect if casein phosphate is a true chemical compound.

It was not possible to plot the corresponding curves for casein sulfate and casein oxalate since these salts are too sparingly soluble. This is true also for casein salts with other acids; *e.g.*, triacetic acid.

3. The writer had shown that the influence of different acids on the physical properties of gelatin or crystalline egg albumin depends only upon the valency and not upon the nature of the ion in combination with the protein.[3] Thus the values of osmotic pressure or viscosity of gelatin chloride are identical with those of gelatin phosphate for

[3] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 559; 1920–21, iii, 85, 247; *Science*, 1920, lii, 449.

the same pH and the same concentration of originally isoelectric gelatin; and the same is true for crystalline egg albumin. The reason is that in the case of gelatin or albumin phosphate the anion is the

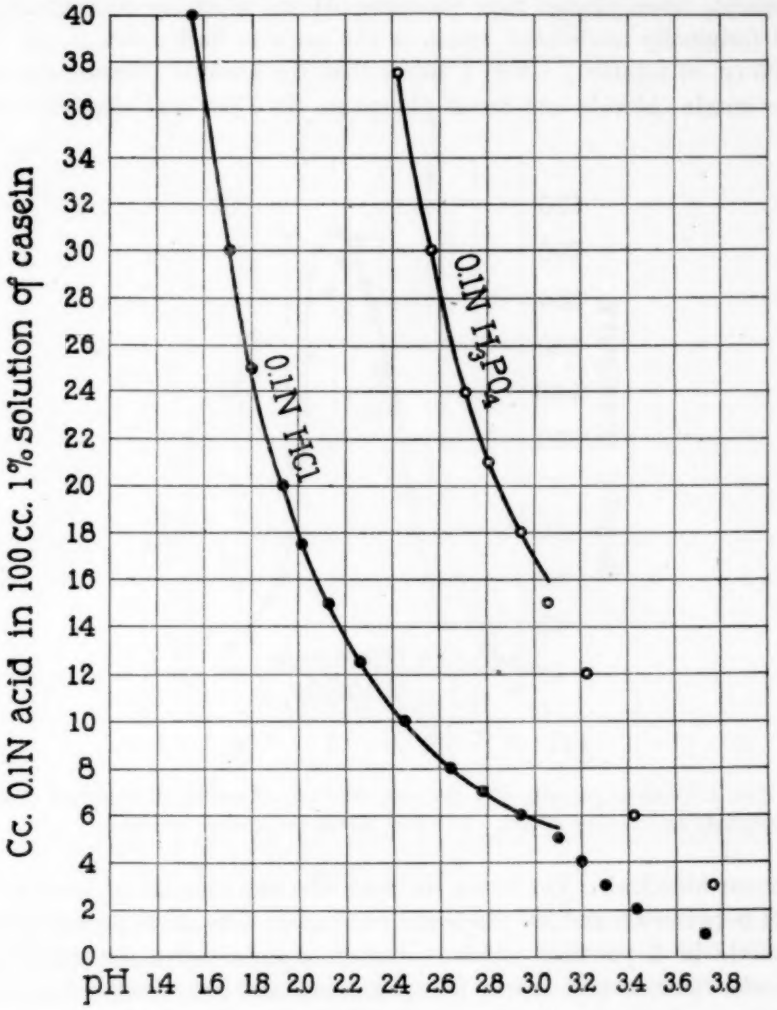

FIG. 1. Ordinates represent the cc. of 0.1 N HCl or H_3PO_4 in 100 cc. of 1 per cent casein solution. The abscissæ are the pH of the solution. Approximately three times as many cc. of 0.1 N H_3PO_4 as of 0.1 N HCl are required to bring 1 gm. of casein to the same pH.

monovalent anion H_2PO_4 and not the trivalent anion PO_4. If the same rule holds for casein, the osmotic pressure and viscosity of casein phosphate should be practically identical with that of casein chloride when plotted over the same pH and when the concentration of (originally isoelectric) casein is the same in both cases (1 gm. in 100 cc. of solution). Fig. 2 shows that the osmotic pressure curves for casein chloride and casein phosphate (in 1 per cent solutions) are

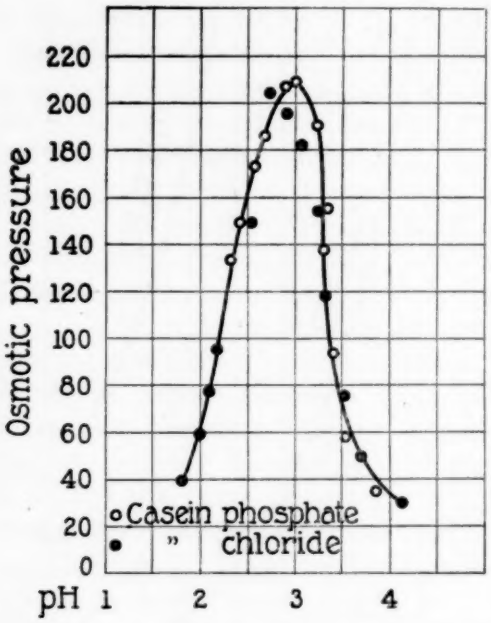

FIG. 2. Osmotic pressure of 1 per cent solutions of casein chloride and casein phosphate as function of pH. The two curves are almost identical.

almost identical. The curve includes also the osmotic pressure at a pH between 4.0 and 3.0 where the two casein salts are not completely soluble in 1 per cent solutions, but since the relative solubilities of casein chloride and casein phosphate are also practically identical, the osmotic pressure curves for the pH where the solubility of the two salts is not complete remain approximately the same.

Fig. 3 gives the viscosity curve for 1 per cent solutions of casein chloride and casein phosphate over pH as abscissæ. The ordinates

are the quotients of the time of outflow of the casein solutions over the time of outflow of distilled water through the same viscometer. The time of outflow for distilled water was 60 seconds. We will, for the sake of brevity, call this ratio specific viscosity. We see that this specific viscosity does not rise above 1 as long as the pH is above

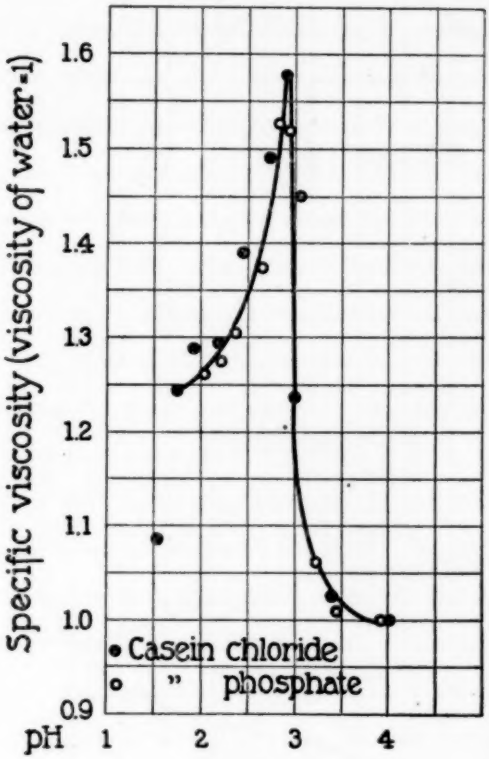

FIG. 3. Viscosity of 1 per cent solutions of casein chloride and casein phosphate. The curves are approximately identical.

3.5. At a pH of 3.0 a sharp rise occurs, because the solubility of the casein increases at this point considerably. With a further fall of pH the viscosity diminishes again. Fig. 3 shows that the viscosity curves for casein chloride and casein phosphate are almost identical, which was to be expected if the rules found for gelatin and crystalline egg albumin are also true for casein.

We had shown that when we add $Ca(OH)_2$ or $Ba(OH)_2$ to isoelectric gelatin or isoelectric crystalline egg albumin the two alkalies combine with the protein in equivalent proportion.[3] Hence the same number

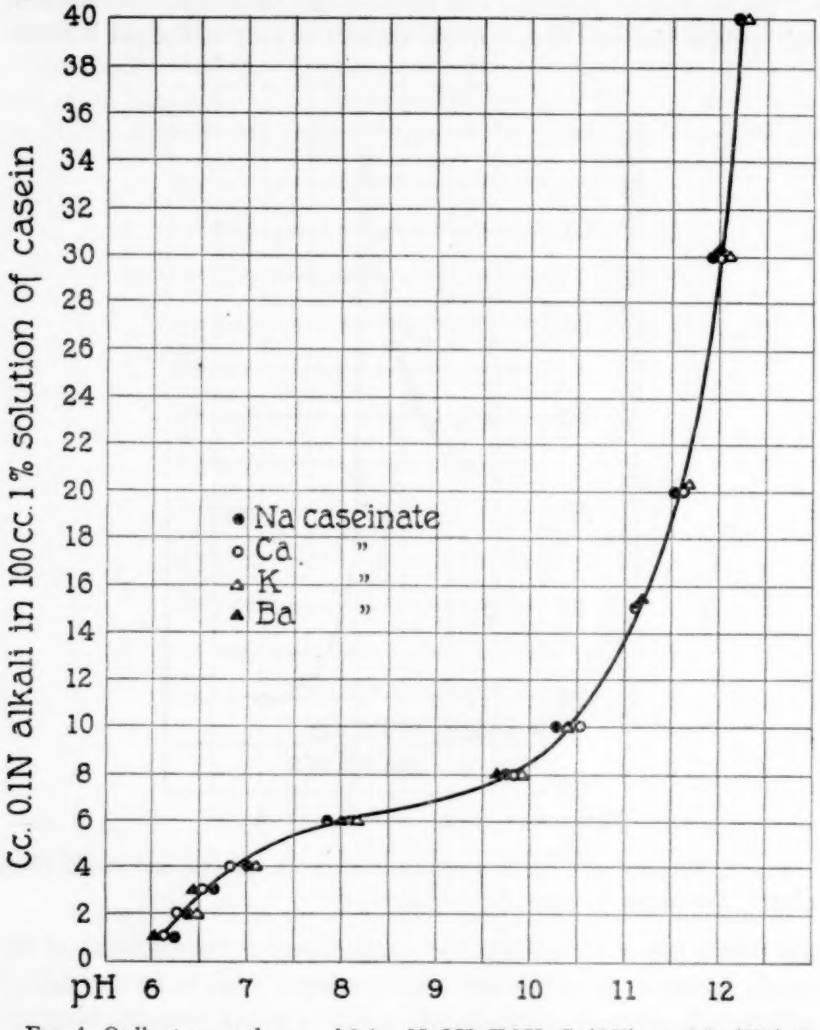

FIG. 4. Ordinates are the cc. of 0.1 N NaOH, KOH, $Ca(OH)_2$, and $Ba(OH)_2$ in 100 cc. of 1 per cent solution of casein. Abscissæ are the pH of the solution. The curves for the four alkalies are identical, proving that Ba and Ca combine with casein in equivalent proportion.

of cc. of 0.1 N $Ba(OH)_2$ or $Ca(OH)_2$ was required to bring a 1 per cent solution of isoelectric gelatin or crystalline egg albumin to a given pH as was required in the case of NaOH or KOH. It can be shown

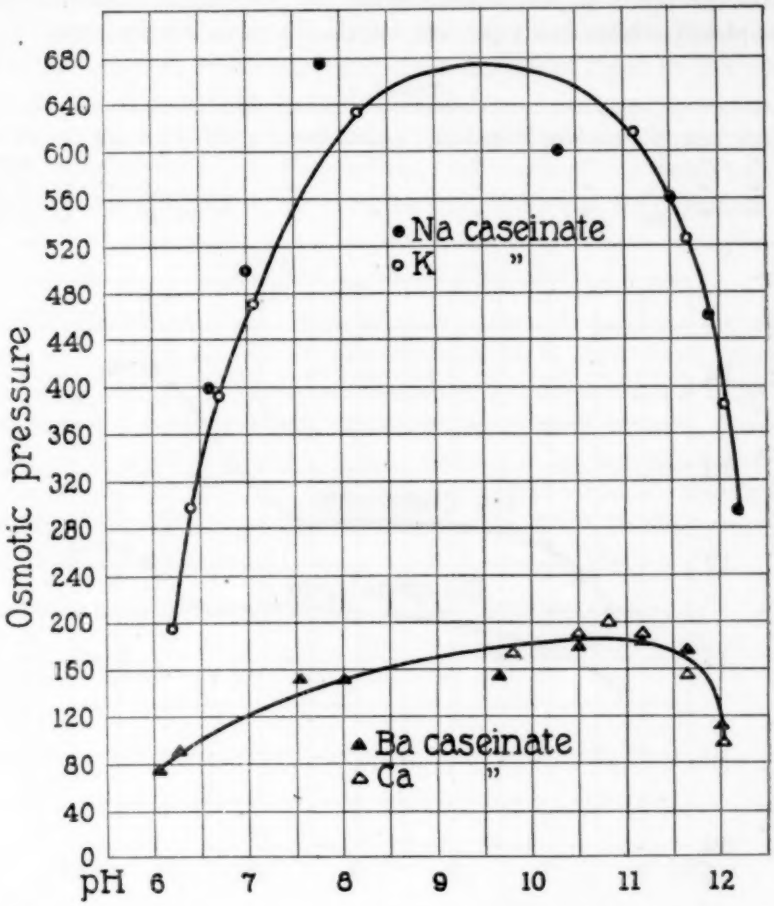

FIG. 5. Osmotic pressure of Na caseinate, K caseinate, Ba caseinate, and Ca caseinate. The curves for the former two caseinates are identical and considerably higher than those for the latter two caseinates.

that the same is true for casein. In Fig. 4 the abscissæ are the pH, the ordinates the number of cc. of 0.1 N NaOH, KOH, $Ca(OH)_2$, and $Ba(OH)_2$ that must be contained in 100 cc. of a 1 per cent solution

of isoelectric casein to bring it to the same pH. The curves for the four alkalies are identical (Fig. 4), thus proving that $Ca(OH)_2$ and $Ba(OH)_2$ combine with casein in equivalent proportion. Table I gives the limits of pH between which the four metal caseinates are completely soluble in a 1 per cent solution at room temperature.

TABLE I.

1	per	cent	Na	caseinate	completely	soluble	between	pH	7.02	and	> 12.20
1	"	"	K	"	"	"	"	"	7.09	"	> 12.28
1	"	"	Ca	"	"	"	"	"	10.53	"	> 12.00
1	"	"	Ba	"	"	"	"	"	10.50	"	> 12.26

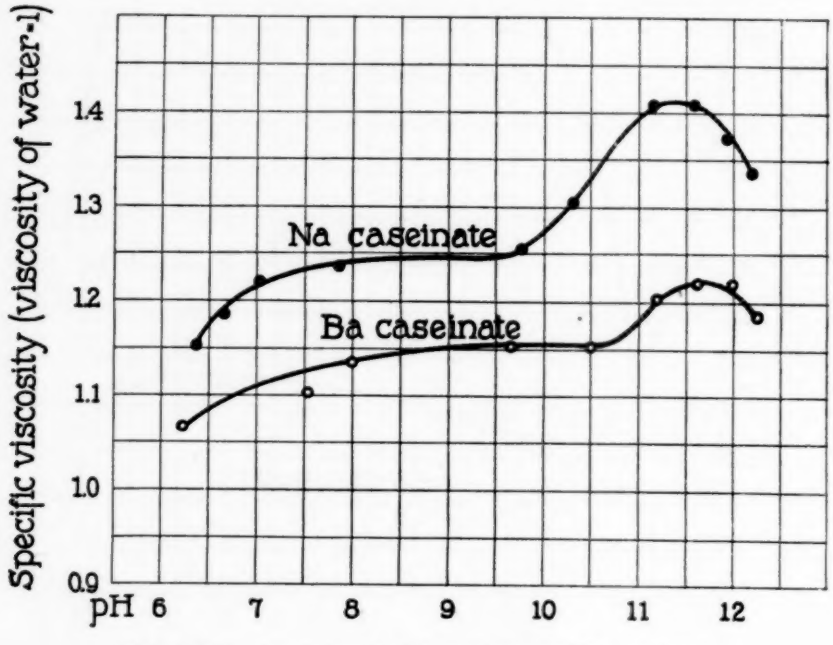

FIG. 6. Viscosity curves for Na caseinate and Ba caseinate.

On account of the incomplete solubility of Ba caseinate and Ca caseinate at a pH between 4.7 and 10.5 it is not possible to draw any conclusion from the relative osmotic pressure or relative viscosity of metal caseinates with monovalent and divalent cation between a pH of 4.7 and 10.5. This should be kept in mind in judging the curves in Figs. 5 and 6.

When we plot the osmotic pressure of these solutions as ordinates over the pH as abscissæ (Fig. 5), we notice that the curves for the osmotic pressure of Na and K caseinate are alike; the curves for the casein salts are, however, over three times as high when the cation is monovalent (Na or K) than when the cation is bivalent (Ca or Ba). This is, however, chiefly the result of the fact that Ca and Ba caseinate are incompletely soluble up to a pH of 10.5.

Fig. 6 gives the viscosity curves for Na caseinate and Ba caseinate. The difference in height between the two casein salts is between pH 11 and 12 of a similar order as in the case of Na and Ba gelatinate.

SUMMARY AND CONCLUSION.

The experiments on casein solutions therefore confirm the conclusion at which we arrived from the behavior of gelatin and crystalline egg albumin that the forces determining the combination between proteins and acids or alkalies are the same forces of primary valency which also determine the reaction between acids and alkalies with crystalloids, and that the valency and not the nature of the ion in combination with a protein determines the effect on the physical properties of the protein.

The measurements mentioned in this paper were made by Dr. E. Brakeley, Mr. M. Kunitz, and Mr. N. Wuest of this Laboratory, to whom I wish to express my indebtedness.

THE COLLOIDAL BEHAVIOR OF PROTEINS.

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I.

Colloids show a number of peculiarities which at first appearance seem to be lacking in crystalloids, and these properties are generally accounted for by differences in the degree of dispersion. We have repeatedly discussed these peculiarities in our analysis of the chemical behavior of proteins, *e.g.* the depressing effect of neutral salts on the osmotic pressure, swelling, and viscosity of certain proteins; the peculiar influence of the hydrogen ion concentration of the solution on these properties; and finally the peculiar influence of the valency of the ion with which the protein is in combination and the apparent lack of influence of the other chemical properties of the ion except valency and sign of charge.[1] The dispersion theory accounts for these difficulties by the assumption of differences in the degree of aggregation of the protein particles. If, for example, the addition of some salt to a protein solution of a definite pH lowers its osmotic pressure, the assumption is made that the salt diminishes the degree of dispersion of the colloidal particles in solution. It is not possible to submit the dispersion theory to a quantitative test since we cannot measure the degree of dispersion of a protein.

A second theory to account for the influence of salts and hydrogen ion concentration is Pauli's ionization theory which ascribes the osmotic pressure, the swelling, and viscosity chiefly to the hydratation of ionized protein, while the non-ionized protein molecule is assumed not to be hydrated. The idea of such a hydratation of protein ions has become doubtful in view of recent experimental and theoretical investigations by Lorenz[2] and by Born,[3] and, moreover, the writer

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85, 247, 391; *Science*, 1920, lii, 449.

[2] Lorenz, R., *Z. Elektrochem.*, 1920, xxvi, 424.

[3] Born, M., *Z. Elektrochem.*, 1920, xxvi, 401.

has been able to show that the conductivity measurements of protein solutions contradict Pauli's ionization theory.

The solution of the problem seems to lie in a field altogether foreign to the speculations current in colloid chemistry, namely in the Donnan equilibrium, which exists when a membrane separates two solutions, one of an electrolyte for the ions of which the membrane is permeable, and one of an electrolyte for one ion of which the membrane is not permeable.[4] It is immaterial whether the latter ion is a colloid or a crystalloid; it is only necessary that it cannot diffuse through the membrane. When a collodion membrane separates a gelatin or albumin chloride solution of pH 3.3 from an aqueous solution of HCl of originally the same pH (but without gelatin), the pH is no longer the same on both sides of the membrane at the time of equilibrium but is lower on the outside than in the gelatin solution. The Donnan equilibrium demands in this case that acid be given off from the gelatin chloride solution to the outside aqueous solution (containing no gelatin). The writer has found that, *e.g.* a 1 per cent gelatin chloride solution of pH 3.5 is in equilibrium with an aqueous HCl solution of about pH 3.0.[5]

II.

Gelatin chloride solutions containing 1 gm. of originally isoelectric gelatin in 100 cc. solution and having a pH of 3.5 were made up in H_2O and in different concentrations of $NaNO_3$ varying from M/4,096 to M/32 $NaNO_3$, all of pH of 3.5. These solutions were put into collodion bags connected with a manometer to measure the final osmotic pressure of the solution. The collodion bags were put into HCl solution of pH 3.0 made up in water and different concentrations of $NaNO_3$, the pH of the $NaNO_3$ solutions being also 3.0. These outside solutions contained no gelatin. The collodion bags were put into these aqueous solutions free from gelatin in such a way that the concentration of the $NaNO_3$ solution inside the collodion bag was always the

[4] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 247.

same as outside. The osmotic pressure of the 1 per cent gelatin chloride solution, which was after 18 hours 435 mm. when no salt was present, was only 63 mm. when the inside and outside solutions were made up in M/32 $NaNO_3$.

Table I gives the influence of the concentration of $NaNO_3$ on the osmotic pressure. It is obvious that the osmotic pressure diminishes with the concentration of the salt. This phenomenon had already been described by Lillie[6] and by the writer.[7] Donnan has shown that this depressing effect of a salt on the osmotic pressure of a colloidal solution is a necessary consequence of his theory of membrane equilibrium, and this conclusion is supported by the following experiments.

TABLE I.

Original inside solution, 1 per cent gelatin chloride of pH 3.5 made up in various concentrations of $NaNO_3$ of the same pH.

Outside solution, HCl of pH 3.0 made up in the same concentrations of $NaNO_3$ of pH 3.0 as the inside solution.

	Concentration of $NaNO_3$.								
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32
Osmotic pressure in mm. of H_2O..	435	405	371	335	280	215	134	85	63
P.D. inside solution in millivolts.....	+31	+26	+24	+22	+16	+12	+7	+4	0

The writer undertook measurements of the potential difference between the gelatin chloride solutions inside the collodion bag and the aqueous solutions outside the collodion bag with the aid of a Compton electrometer. It was found that the gelatin chloride solution was always positively charged while the outside aqueous solution was negatively charged, as was to be expected. The second important fact is that the P.D. diminishes with the increase in concentration of the neutral salt added and (if the necessary corrections are made) in approximately the same ratio as the osmotic pressure diminishes (lower row in Table I).

[6] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127.

[7] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 391.

III.

What is the origin of these potential differences? Beutner[8] has shown that the potential differences at the boundary of water and water-immiscible substances obey Nernst's well-known logarithmic formula

$$E = \frac{RT}{nF} \ln \frac{C_1}{C_2}$$

or at room temperature and for $n = 1$

$$0.058 \log \frac{C_1}{C_2}$$

Loeb and Beutner have found the same formula to hold for the potential differences at the boundary of water and the skin of an apple, a tomato, or the leaf of a rubber plant.

It can be shown that the potential differences mentioned in Table I follow Nernst's formula, if we assume that only the hydrogen ion concentration need be considered for the potential difference. If C_1 is the concentration of free HCl in the gelatin solution, and C_2 the concentration of HCl in the outside aqueous solution (without gelatin) the value $\log \frac{C_1}{C_2}$ becomes equal to (pH inside − pH outside).

We measured the pH of the gelatin chloride solution (inside solution) and of the outside HCl solution (without gelatin) after the osmotic and the Donnan equilibria were established. The surprising result was noticed that the difference of pH inside the gelatin solution minus the pH of the outside solution (without gelatin) becomes the smaller the greater the concentration of the $NaNO_3$, as shown in Table II.

We can calculate from this difference of pH inside minus pH outside the P.D. between inside and outside solution in millivolts by multiplying the differences by 58 or 59 (correcting for temperature of 24°C.). If the Nernst formula holds the values for P.D. thus calculated should be identical with the observed values for P.D. Table III shows that this is true to a remarkable degree.

[8] Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920.

TABLE II.

pH Inside Minus pH Outside after 18 Hours.

Original inside solution, 1 per cent originally isoelectric gelatin dissolved in various concentrations of $NaNO_3$ made up with HCl to pH 3.5.

Outside solution, same concentrations of $NaNO_3$ all made up with HCl to pH 3.0.

	Concentration of $NaNO_3$.								
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32
pH of inside solution.....	3.58	3.56	3.51	3.46	3.41	3.36	3.32	3.29	3.25
" " outside "	3.05	3.08	3.10	3.11	3.14	3.17	3.20	3.22	3.24
Difference pH inside minus pH outside.....	0.53	0.48	0.41	0.35	0.27	0.19	0.12	0.07	0.01

TABLE III.

Potential Difference between Gelatin Solution and Outside Solution.

Concentration of $NaNO_3$.	Calculated by Nernst's formula from pH.	Observed.
	millivolts	*millivolts*
0	31.2	31
M/4,096	28.3	28
M/2,048	24.0	24
M/1,024	20.7	22
M/512	16.0	16
M/256	11.2	12
M/128	7.0	7
M/64	4.1	4
M/32	0	0

IV.

The greatest puzzle in the physical chemistry of the proteins is the fact that at the isoelectric point of gelatin and crystalline egg albumin the osmotic pressure is a minimum, that it rises when acid is added, at first with the increase of acid added, reaching a maximum when the pH is about 3.5, and that on further addition of acid a rapid fall of osmotic pressure occurs.

It was also reported in the preceding papers that the osmotic pressure of gelatin chloride and gelatin phosphate solutions of the same pH and the same concentration of originally isoelectric gelatin was about the same, that the osmotic pressure of gelatin oxalate was slightly

lower, while that of gelatin sulfate was only half as high or not quite half as high as that of gelatin chloride.

Measurements of the potential differences between the gelatin solution and the outside solution revealed the fact that the curves presenting the P.D. as a function of the hydrogen ion concentration resemble the curves for osmotic pressure. The P.D. curves have a minimum at the isoelectric point, rise steeply with increasing hydrogen ion concentration until a pH of 3.9 is reached, then drop equally steeply again when the pH falls further. Moreover, the maximum of the P.D. curve for gelatin sulfate is about one-half of that of the maximum of the P.D. curve for gelatin chloride and the P.D. curve for gelatin chloride is about equal to the P.D. curve for gelatin phosphate, while that of gelatin oxalate is only slightly lower than that of gelatin chloride.

The next question was whether or not the Nernst formula can account for these differences. The pH of the gelatin solutions and of the outside solutions were measured at the point of equilibrium and it was found that the difference of pH inside minus pH outside multiplied by 58 gave approximately the number of millivolts actually measured. The agreement between the calculated P.D. and the observed P.D. was not as perfect as in the case of the salt effect (Table III) but sufficient to leave no doubt that Nernst's theory accounts for these P.D.

V.

Procter[9] has applied the Donnan equilibrium to the theory of swelling of gelatin, reaching the conclusion that swelling is an osmotic phenomenon and that the amount of swelling of a gelatin chloride solution is determined by the concentration of the free ions inside the gel minus the concentration of the free ions in the outside solution. He did not measure the pH. By filling this gap the writer was able to satisfy himself that the depressing influence of salts upon the swelling of gelatin is due to a diminution of the difference of pH inside

[9] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

and outside the gel, and that the curves expressing the influence of neutral salts on the value of pH inside minus pH outside the gel, and on the swelling run approximately parallel.

SUMMARY AND CONCLUSION.

1. It is well known that neutral salts depress the osmotic pressure, swelling, and viscosity of protein-acid salts. Measurements of the P.D. between gelatin chloride solutions contained in a collodion bag and an outside aqueous solution show that the salt depresses the P.D. in the same proportion as it depresses the osmotic pressure of the gelatin chloride solution.

2. Measurements of the hydrogen ion concentration inside the gelatin chloride solution and in the outside aqueous solution show that the difference in pH of the two solutions allows us to calculate the P.D. quantitatively on the basis of the Nernst formula $E = \frac{RT}{nF} ln \frac{C_1}{C_2}$ if we assume that the P.D. is due to a difference in the hydrogen ion concentration on the two sides of the membrane.

3. This difference in pH inside minus pH outside solution seems to be the consequence of the Donnan membrane equilibrium, which only supposes that one of the ions in solution cannot diffuse through the membrane. It is immaterial for this equilibrium whether the non-diffusible ion is a crystalloid or a colloid.

4. When acid is added to isoelectric gelatin the osmotic pressure rises at first with increasing hydrogen ion concentration, reaches a maximum at pH 3.5, and then falls again with further fall of the pH. It is shown that the P.D. of the gelatin chloride solution shows the same variation with the pH (except that it reaches its maximum at pH of about 3.9) and that the P.D. can be calculated from the difference of pH inside minus pH outside on the basis of Nernst's formula.

5. It was found in preceding papers that the osmotic pressure of gelatin sulfate solutions is only about one-half of that of gelatin chloride or gelatin phosphate solutions of the same pH and the same concentration of originally isoelectric gelatin; and that the osmotic pressure of gelatin oxalate solutions is almost but not quite the same as that of the gelatin chloride solutions of the same pH and concentra-

tion of originally isoelectric gelatin. It was found that the curves for the values for P.D. of these four gelatin salts are parallel to the curves of their osmotic pressure and that the values for pH inside minus pH outside multiplied by 58 give approximately the millivolts of these P.D.

In this preliminary note only the influence of the concentration of the hydrogen ions on the P.D. has been taken into consideration. In the fuller paper, which is to follow, the possible influence of the concentration of the anions on this quantity will have to be discussed.

The writer wishes to express his indebtedness to his technical assistants, Mr. M. Kunitz and Mr. N. Wuest, who have carried out the measurements in these experiments.